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(54) Title: TARGETED MULTIFUNCTIONAL PROTEINS

(57) Abstract

Disclosed are a family of synthetic proteins having binding affinity for a preselected antigen, and multifunctional proteins having such affinity. The proteins are characterized by one or more sequences of amino acids constituting a region which behaves as a biosynthetic antibody binding site (BABS). The sites comprise V_H-V_L or V_L-V_H-like single chains wherein the V_H and V_L-like sequences are attached by a polypeptide linker, or individual V_H or V_L-like domains. The binding domains comprise linked CDR and FR regions, which may be derived from separate immunoglobulins. The proteins may also include other polypeptide sequences which function, e.g., as an enzyme, toxin, binding site, or site for attachment to an immobilization media or radioactive atom. Methods are disclosed for producing the proteins, for designing BABS having any specificity that can be elicited by *in vivo* generation of antibody, for producing analogs thereof, and for producing multifunctional synthetic proteins which are self-targeted by virtue of their binding site region.

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TARGETED MULTIFUNCTIONAL PROTEINS

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Reference to Related Applications

This application is a continuation-in-part of copending U.S. application serial number 052,800 filed May 21, 1987, the disclosure of which is incorporated herein by reference.

Background of the Invention

This invention relates to novel compositions of matter, hereinafter called targeted multifunctional proteins, useful, for example, in specific binding assays, affinity purification, biocatalysis, drug targeting, imaging, immunological treatment of various oncogenic and infectious diseases, and in other contexts. More specifically, this invention relates to biosynthetic proteins expressed from recombinant DNA as a single polypeptide chain comprising plural regions, one of which has a structure similar to an antibody binding site, and an affinity for a preselected antigenic determinant, and another of which has a separate function, and may be biologically active, designed to

bind to ions, or designed to facilitate immobilization of the protein. This invention also relates to the binding proteins per se, and methods for their construction.

There are five classes of human antibodies. Each has the same basic structure (see Figure 1), or multiple thereof, consisting of two identical polypeptides called heavy (H) chains (molecularly weight approximately 50,000 d) and two identical light (L) chains (molecular weight approximately 25,000 d). Each of the five antibody classes has a similar set of light chains and a distinct set of heavy chains. A light chain is composed of one variable and one constant domain, while a heavy chain is composed of one variable and three or more constant domains. The combined variable domains of a paired light and heavy chain are known as the Fv region, or simply "Fv". The Fv determines the specificity of the immunoglobulin, the constant regions have other functions.

Amino acid sequence data indicate that each variable domain comprises three hypervariable regions or loops, sometimes called complementarity determining regions or "CDRs" flanked by four relatively conserved framework regions or "FRs" (Kabat et. al., <u>Sequences of Proteins of Immunological Interest</u> [U.S. Department of Health and Human Services, third edition, 1983, fourth edition, 1987]). The hypervariable regions have been assumed to be responsible for the binding specificity of individual antibodies and to account for the diversity of binding of antibodies as a protein class.

Monoclonal antibodies have been used both as diagnostic and therapeutic agents. They are routinely produced according to established procedures by hybridomas generated by fusion of mouse lymphoid cells with an appropriate mouse myeloma cell line.

The literature contains a host of references to the concept of targeting bioactive substances such as drugs, toxins, and enzymes to specific points in the body to destroy or locate malignant cells or to induce a localized drug or enzymatic effect. It has been proposed to achieve this effect by conjugating the bioactive substance to monoclonal antibodies (see, e.g., Vogel, Immunoconjugates. Antibody Conjugates in Radioimaging and Therapy of Cancer, 1987, N.Y., Oxford University Press; and Ghose et al. (1978) J. Natl. Cancer Inst. 61:657-676,). However, non-human antibodies induce an immune response when injected into humans. Human monoclonal antibodies may alleviate this problem, but they are difficult to produce by cell fusion techniques since, among other problems, human hybridomas are notably unstable, and removal of immunized spleen cells from humans is not feasible.

Chimeric antibodies composed of human and non-human amino acid sequences potentially have improved therapeutic value as they presumably would elicit less circulating human antibody against the non-human immunoglobulin sequences. Accordingly, hybrid antibody molecules have been proposed which consist of amino acid sequences from different mammalian sources. The chimeric antibodies designed

thus far comprise variable regions from one mammalian source, and constant regions from human or another mammalian source (Morrison et al. (1984) Proc. Natl. Acad. Sci. U.S.A., 81:5851-6855; Neuberger et al. (1984) Nature 312:604-608; Sahagan et al. (1986) J. Immunol. 137:1066-1074; EPO application nos. 84302368.0, Genentech; 85102665.8, Research Development Corporation of Japan; 85305604.2, Stanford; P.C.T. application no. PCT/GB85/00392, Celltech Limited).

It has been reported that binding function is localized to the variable domains of the antibody molecule located at the amino terminal end of both the heavy and light chains. The variable regions remain noncovalently associated (as $V_H V_L$ dimers, termed Fv regions) even after proteolytic cleavage from the native antibody molecule, and retain much of their antigen recognition and binding capabilities (see, for example, Inbar et al., Proc. Natl. Acad. Sci. U.S.A. (1972) 69:2659-2662; Hochman et. al. (1973) Biochem. 12:1130-1135; and (1976) Biochem. 15:2706-2710; Sharon and Givol (1976) Biochem. 15:1591-1594; Rosenblatt and Haber (1978) Biochem. 17:3877-3882; Ehrlich et al. (1980) Biochem. 19:4091-40996). Methods of manufacturing two-chain Fv substantially free of constant region using recombinant DNA techniques are disclosed in U.S. 4,642,334 and corresponding published specification EP 088,994.

Summary of the Invention

In one aspect the invention provides a single chain multifunctional biosynthetic protein expressed from a single gene derived by recombinant DNA techniques. The protein comprises a biosynthetic antibody binding site (BABS) comprising at least one protein domain capable of binding to a preselected antigenic determinant. The amino acid sequence of the domain is homologous to at least a portion of the sequence of a variable region of an immunoglobulin molecule capable of binding the preselected antigenic determinant. Peptide bonded to the binding site is a polypeptide consisting of an effector protein having a conformation suitable for biological activity in a mammal, an amino acid sequence capable of sequestering ions, or an amino acid sequence capable of selective binding to a solid support.

In another aspect, the invention provides biosynthetic binding site protein comprising a single polypeptide chain defining two polypeptide domains connected by a polypeptide linker. The amino acid sequence of each of the domains comprises a set of complementarity determining regions (CDRs) interposed between a set of framework regions (FRs), each of which is respectively homologous with at least a portion of the CDRs and FRS from an immunoglobulin molecule. At least one of the domains comprises a set of CDR amino acid sequences and a set of FR amino acid sequences at least partly homologous to different immunoglobulins. The two polypeptide

domains together define a hybrid synthetic binding site having specificity for a preselected antigen, determined by the selected CDRs.

In still another aspect, the invention provides biosynthetic binding protein comprising a single polypeptide chain defining two domains connected by a polypeptide linker. The amino acid sequence of each of the domains comprises a set of CDRs interposed between a set of FRs, each of which is respectively homologous with at least a portion of the CDRs and FRs from an immunoglobulin molecule. The linker comprises plural, peptide-bonded amino acids defining a polypeptide of a length sufficient to span the distance between the C terminal end of one of the domains and N terminal end of the other when the binding protein assumes a conformation suitable for binding. The linker comprises hydrophilic amino acids which together preferably constitute a hydrophilic sequence. Linkers which assume an unstructured polypeptide configuration in aqueous solution work well. The binding protein is capable of binding to a preselected antigenic site, determined by the collective tertiary structure of the sets of CDRs held in proper conformation by the sets of FRs. Preferably, the binding protein has a specificity at least substantially identical to the binding specificity of the immunoglobulin molecule used as a template for the design of the CDR regions. Such structures can have a binding affinity of at least 10^6, M^{-1}, and preferably 10^8 M^{-1}.

In preferred aspects, the FRs of the binding protein are homologous to at least a portion of the FRs from a human immunoglobulin, the linker spans at

least about 40 angstroms; a polypeptide spacer is incorporated in the multifunctional protein between the binding site and the second polypeptide; and the binding protein has an affinity for the preselected antigenic determinant no less than two orders of magnitude less than the binding affinity of the immunoglobulin molecule used as a template for the CDR regions of the binding protein. The preferred linkers and spacers are cysteine-free. The linker preferably comprises amino acids having unreactive side groups, e.g., alanine and glycine. Linkers and spacers can be made by combining plural consecutive copies of an amino acid sequence, e.g., $(Gly_4 Ser)_3$. The invention also provides DNAs encoding these proteins and host cells harboring and capable of expressing these DNAs.

As used herein, the phrase biosynthetic antibody binding site or BABS means synthetic proteins expressed from DNA derived by recombinant techniques. BABS comprise biosynthetically produced sequences of amino acids defining polypeptides designed to bind with a preselected antigenic material. The structure of these synthetic polypeptides is unlike that of naturally occurring antibodies, fragments thereof, e.g., Fv, or known synthetic polypeptides or "chimeric antibodies" in that the regions of the BABS responsible for specificity and affinity of binding, (analogous to native antibody variable regions) are linked by peptide bonds, expressed from a single DNA, and may themselves be chimeric, e.g., may comprise amino acid sequences homologous to portions of at least two

different antibody molecules. The BABS embodying the invention are biosynthetic in the sense that they are synthesized in a cellular host made to express a synthetic DNA, that is, a recombinant DNA made by ligation of plural, chemically synthesized oligonucleotides, or by ligation of fragments of DNA derived from the genome of a hybridoma, mature B cell clone, or a cDNA library derived from such natural sources. The proteins of the invention are properly characterized as "binding sites" in that these synthetic molecules are designed to have specific affinity for a preselected antigenic determinant. The polypeptides of the invention comprise structures patterned after regions of native antibodies known to be responsible for antigen recognition.

Accordingly, it is an object of the invention to provide novel multifunctional proteins comprising one or more effector proteins and one or more biosynthetic antibody binding sites, and to provide DNA sequences which encode the proteins. Another object is to provide a generalized method for producing biosynthetic antibody binding site polypeptides of any desired specificity.

Brief Description of the Drawing

The foregoing and other objects of this invention, the various features thereof, as well as the invention itself, may be more fully understood from the following description, when read together with the accompanying drawings.

Figure 1A is a schematic representation of an intact IgG antibody molecule containing two light chains, each consisting of one variable and one constant domain, and two heavy chains, each consisting of one variable and three constant domains. Figure 1B is a schematic drawing of the structure of Fv proteins (and DNA encoding them) illustrating V_H and V_L domains, each of which comprises four framework (FR) regions and three complementarity determining (CDR) regions. Boundaries of CDRs are indicated, by way of example, for monoclonal 26-10, a well known and characterized murine monoclonal specific for digoxin.

Figure 2A-2E are schematic representations of some of the classes of reagents constructed in accordance with the invention, each of which comprises a biosynthetic antibody binding site.

Figure 3 discloses five amino acid sequences (heavy chains) in single letter code lined up vertically to facilitate understanding of the invention. Sequence 1 is the known native sequence

of V_H from murine monoclonal glp-4 (anti-lysozyme). Sequence 2 is the known native sequence of V_H from murine monoclonal 26-10 (anti-digoxin). Sequence 3 is a BABS comprising the FRs from 26-10 V_H and the CDRs from glp-4 V_H. The CDRs are identified in lower case letters; restriction sites in the DNA used to produce chimeric sequence 3 are also identified. Sequence 4 is the known native sequence of V_H from human myeloma antibody NEWM. Sequence 5 is a BABS comprising the FRs from NEWM V_H and the CDRs from glp-4 V_H, i.e., illustrates a "humanized" binding site having a human framework but an affinity for lysozyme similar to murine glp-4.

Figures 4A-4F are the synthetic nucleic acid sequences and encoded amino acid sequences of (4A) the heavy chain variable domain of murine anti-digoxin monoclonal 26-10; (4B) the light chain variable domain of murine anti-digoxin monoclonal 26-10; (4C) a heavy chain variable domain of a BABS comprising CDRs of glp-4 and FRs of 26-10; (4D) a light chain variable region of the same BABS; (4E) a heavy chain variable region of a BABS comprising CDRs of glp-4 and FRs of NEWM; and (4F) a light chain variable region comprising CDRs of glp-4 and FRs of NEWM. Delineated are FRs, CDRs, and restriction sites for endonuclease digestion, most of which were introduced during design of the DNA.

Figure 5 is the nucleic acid and encoded amino acid sequence of a host DNA (V_H) designed to facilitate insertion of CDRs of choice. The DNA was designed to have unique 6-base sites directly flanking the CDRs so that relatively small oligonucleotides defining portions of CDRs can be readily inserted, and to have other sites to facilitate manipulation of the DNA to optimize binding properties in a given construct. The framework regions of the molecule correspond to murine FRs (Figure 4A).

Figures 6A and 6B are multifunctional proteins (and DNA encoding them) comprising a single chain BABS with the specificity of murine monoclonal 26-10, linked through a spacer to the FB fragment of protein A, here fused as a leader, and constituting a binding site for Fc. The spacer comprises the 11 C-terminal amino acids of the FB followed by Asp-Pro (a dilute acid cleavage site). The single chain BABS comprises sequences mimicking the V_H and V_L (6A) and the V_L and V_H (6B) of murine monoclonal 26-10. The V_L in construct 6A is altered at residue 4 where valine replaces methionine present in the parent 26-10 sequence. These constructs contain binding sites for both Fc and digoxin. Their structure may be summarized as;

(6A) FB-Asp-Pro-V_H-$(Gly_4\text{-}Ser)_3$-V_L,

and

(6B) FB-Asp-Pro-V_L-$(Gly_4\text{-}Ser)_3$-V_H,

where $(Gly_4\text{-}Ser)_3$ is a polypeptide linker.

In Figures 4A-4E and 6A and 6B, the amino acid sequence of the expression products start after the GAATTC sequences, which codes for an EcoRI splice site, translated as Glu-Phe on the drawings.

Figure 7A is a graph of percent of maximum counts bound of radioiodinated digoxin versus concentration of binding protein adsorbed to the plate comparing the binding of native 26-10 (curve 1) and the construct of Figure 6A and Figure 2B renatured using two different procedures (curves 2 and 3). Figure 7B is a graph demonstrating the bifunctionality of the FB-(26-10) BABS adhered to microtiter plates through the specific binding of the binding site to the digoxin-BSA coat on the plate. Figure 7B shows the percent inhibition of ^{125}I-rabbit-IgG binding to the FB domain of the FB BABS by the addition of IgG, protein A, FB, murine IgG2a, and murine IgG1.

Figure 8 is a schematic representation of a model assembled DNA sequence encoding a multifunctional biosynthetic protein comprising a leader peptide (used to aid expression and thereafter cleaved), a binding site, a spacer, and an effector molecule attached as a trailer sequence.

Figure 9A-9E are exemplary synthetic nucleic acid sequences and corresponding encoded amino acid sequences of binding sites of different specificities: (A) FRs from NEWM and CDRs from 26-10 having the digoxin specificity of murine monoclonal 26-10; (B) FRs from 26-10, and CDRs from G-loop-4

(glp-4) having lysozyme specificity; (C) FRs and CDRs from MOPC-315 having dinitrophenol (DNF) specificity; (D) FRs and CDRs from an anti-CEA monoclonal antibody; (E) FRs in both V_H and V_L and CDR_1 and CDR_3 in V_H, and CDR_1, CDR_2, and CDR_3 in V_L from an anti-CEA monoclonal antibody; CDR_2 in V_H is a CDR_2 consensus sequence found in most immunoglobulin V_H regions.

Figure 10A is a schematic representation of the DNA and amino acid sequence of a leader peptide (MLE) protein with corresponding DNA sequence and some major restriction sites. Figure 10B shows the design of an expression plasmid used to express MLE-BABS (26-10). During construction of the gene, fusion partners were joined at the EcoR1 site that is shown as part of the leader sequence. The pBR322 plasmid, opened at the unique SspI and PstI sites, was combined in a 3-part ligation with an SspI to EcoRI fragment bearing the <u>trp</u> promoter and MLE leader and with an EcoRI to PstI fragment carrying the BABS gene. The resulting expression vector confers tetracycline resistance on positive transformants.

Figure 11 is an SDS-polyacrylamide gel (15%) of the (26-10) BABS at progressive stages of purification. Lane 0 shows low molecular weight standards; lane 1 is the MLE-BABS fusion protein; lane 2 is an acid digest of this material; lane 3 is the pooled DE-52 chromatographed protein; lanes 4 and

5 are the same oubain-Sepharose pool of single chain BABS except that lane 4 protein is reduced and lane 5 protein is unreduced.

Figure 12 shows inhibition curves for 26-10 BABS and 26-10 Fab species, and indicates the relative affinities of the antibody fragment for the indicated cardiac glycosides.

Figures 13A and 13B are plots of digoxin binding curves. (A) shows 26-10 BABS binding isotherm and Sips plot (inset), and (B) shows 26-10 Fab binding isotherm and Sips plot (inset).

Figure 14 is a nucleic acid sequence and corresponding amino acid sequence of a modified FB dimer leader sequence and various restriction sites.

Figure 15A-15H are nucleic acid sequences and corresponding amino acid sequences of biosynthetic multifunctional proteins including a single chain BABS and various biologically active protein trailers linked via a spacer sequence. Also indicated are various endonuclease digestion sites. The trailing sequences are (A) epidermal growth factor (EGF); (B) streptavidin; (C) tumor necrosis factor (TNF); (D) calmodulin; (E) platelet derived growth factor-beta (PDGF-beta); (F) ricin; and (G) interleukin-2, and (H) an FB-FB dimer.

Description

The invention will first be described in its broadest overall aspects with a more detailed description following.

A class of novel biosynthetic, bi or multifunctional proteins has now been designed and engineered which comprise biosynthetic antibody binding sites, that is, "BABS" or biosynthetic polypeptides defining structure capable of selective antigen recognition and preferential antigen binding, and one or more peptide-bonded additional protein or polypeptide regions designed to have a preselected property. Examples of the second region include amino acid sequences designed to sequester ions, which makes the protein suitable for use as an imaging agent, and sequences designed to facilitate immobilization of the protein for use in affinity chromatography and solid phase immunoassay. Another example of the second region is a bioactive effector molecule, that is, a protein having a conformation suitable for biological activity, such as an enzyme, toxin, receptor, binding site, growth factor, cell differentiation factor, lymphokine, cytokine, hormone, or anti-metabolite. This invention features synthetic, multifunctional proteins comprising these regions peptide bonded to one or more biosynthetic antibody binding sites, synthetic, single chain proteins designed to bind preselected antigenic determinants with high affinity and specificity, constructs containing multiple binding sites linked

together to provide multipoint antigen binding and high net affinity and specificity, DNA encoding these proteins prepared by recombinant techniques, host cells harboring these DNAs, and methods for the production of these proteins and DNAs.

The invention requires recombinant production of single chain binding sites having affinity and specificity for a predetermined antigenic determinant. This technology has been developed and is disclosed herein. In view of this disclosure, persons skilled in recombinant DNA technology, protein design, and protein chemistry can produce such sites which, when disposed in solution, have high binding constants (at least 10^6, preferably 10^8 M^{-1},) and excellent specificity.

The design of the BABS is based on the observation that three subregions of the variable domain of each of the heavy and light chains of native immunoglobulin molecules collectively are responsible for antigen recognition and binding. Each of these subregions, called herein "complementarity determining regions" or CDRs, consists of one of the hypervariable regions or loops and of selected amino acids or amino acid sequences disposed in the framework regions or FRs which flank that particular hypervariable region. It has now been discovered that FRs from diverse species are effective to maintin CDRs from diverse other species in proper conformation so as to achieve true immunochemical binding properties in a biosynthetic protein. It has also been discovered that

biosynthetic domains mimicking the structure of the two chains of an immunoglobulin binding site may be connected by a polypeptide linker while closely approaching, retaining, and often improving their collective binding properties.

The binding site region of the multifunctional proteins comprises at least one, and preferably two domains, each of which has an amino acid sequence homologous to portions of the CDRs of the variable domain of an immunoglobulin light or heavy chain, and other sequence homologous to the FRs of the variable domain of the same, or a second, different immunoglobulin light or heavy chain. The two domain binding site construct also includes a polypeptide linking the domains. Polypeptides so constructed bind a specific preselected antigen determined by the CDRs held in proper conformation by the FRs and the linker. Preferred structures have human FRs, i.e., mimic the amino acid sequence of at least a portion of the framework regions of a human immunoglobulin, and have linked domains which together comprise structure mimicking a V_H-V_L or V_L-V_H immunoglobulin two-chain binding site. CDR regions of a mammalian immunoglobulin, such as those of mouse, rat, or human origin are preferred. In one preferred embodiment, the biosynthetic antibody binding site comprises FRs homologous with a portion of the FRs of a human immunoglobulin and CDRs homologous with CDRs from a mouse or rat immunoglobulin. This type of chimeric polypeptide displays the antigen binding specificity of the mouse or rat immunoglobulin, while its human framework

minimizes human immune reactions. In addition, the chimeric polypeptide may comprise other amino acid sequences. It may comprise, for example, a sequence homologous to a portion of the constant domain of an immunoglobulin, but preferably is free of constant regions (other than FRs).

The binding site region(s) of the chimeric proteins are thus single chain composite polypeptides comprising a structure which in solution behaves like an antibody binding site. The two domain, single chain composite polypeptide has a structure patterned after tandem V_H and V_L domains, but with the carboxyl terminal of one attached through a linking amino acid sequence to the amino terminal of the other. The linking amino acid sequence may or may not itself be antigenic or biologically active. It preferably spans a distance of at least about 40A, i.e., comprises at least about 14 amino acids, and comprises residues which together present a hydrophilic, relatively unstructured region. Linking amino acid sequences having little or no secondary structure work well. Optionally, one or a pair of unique amino acids or amino acid sequences recognizable by a site specific cleavage agent may be included in the linker. This permits the V_H and V_L-like domains to be separated after expression, or the linker to be excised after refolding of the binding site.

Either the amino or carboxyl terminal ends (or both ends) of these chimeric, single chain binding sites are attached to an amino acid sequence which itself is bioactive or has some other function

to produce a bifunctional or multifunctional protein. For example, the synthetic binding site may include a leader and/or trailer sequence defining a polypeptide having enzymatic activity, independent affinity for an antigen different from the antigen to which the binding site is directed, or having other functions such as to provide a convenient site of attachment for a radioactive ion, or to provide a residue designed to link chemically to a solid support. This fused, independently functional section of protein should be distinguished from fused leaders used simply to enhance expression in prokaryotic host cells or yeasts. The multifunctional proteins also should be distinguished from the "conjugates" disclosed in the prior art comprising antibodies which, after expression, are linked chemically to a second moiety.

Often, a series of amino acids designed as a "spacer" is interposed between the active regions of the multifunctional protein. Use of such a spacer can promote independent refolding of the regions of the protein. The spacer also may include a specific sequence of amino acids recognized by an endopeptidase, for example, endogenous to a target cell (e.g., one having a surface protein recognized by the binding site) so that the bioactive effector protein is cleaved and released at the target. The second functional protein preferably is present as a trailer sequence, as trailers exhibit less of a tendency to interfere with the binding behavior of the BABS.

The therapeutic use of such "self-targeted" bioactive proteins offers a number of advantages over conjugates of immunoglobulin fragments or complete antibody molecules: they are stable, less immunogenic and have a lower molecular weight; they can penetrate body tissues more rapidly for purposes of imaging or drug delivery because of their smaller size; and they can facilitate accelerated clearance of targeted isotopes or drugs. Furthermore, because design of such structures at the DNA level as disclosed herein permits ready selection of bioproperties and specificities, an essentially limitless combination of binding sites and bioactive proteins is possible, each of which can be refined as disclosed herein to optimize independent activity at each region of the synthetic protein. The synthetic proteins can be expressed in procaryotes such as E. coli, and thus are less costly to produce than immunoglobulins or fragments thereof which require expression in cultured animal cell lines.

The invention thus provides a family of recombinant proteins expressed from a single piece of DNA, all of which have the capacity to bind specifically with a predetermined antigenic determinant. The preferred species of the proteins comprise a second domain which functions independently of the binding region. In this aspect the invention provides an array of "self-targeted" proteins which have a bioactive function and which deliver that function to a locus determined by the binding site's specificity. It also provides biosynthetic binding proteins having attached

polypeptides suitable for attachment to immobilization matrices which may be used in affinity chromatography and solid phase immunoassay applications, or suitable for attachment to ions, e.g., radioactive ions, which may be used for in vivo imaging.

The successful design and manufacture of the proteins of the invention depends on the ability to produce biosynthetic binding sites, and most preferably, sites comprising two domains mimicking the variable domains of immunoglobulin connected by a linker.

As is now well known, Fv, the minimum antibody fragment which contains a complete antigen recognition and binding site, consists of a dimer of one heavy and one light chain variable domain in noncovalent association (Figure 1A). It is in this configuration that the three complementarity determining regions of each variable domain interact to define an antigen binding site on the surface of the V_H-V_L dimer. Collectively, the six complementarity determining regions (see Figure 1B) confer antigen binding specificity to the antibody. FRs flanking the CDRs have a tertiary structure which is essentially conserved in native immunoglobulins of species as diverse as human and mouse. These FRs serve to hold the CDRs in their appropriate orientation. The constant domains are not required for binding function, but may aid in stabilizing V_H-V_L interaction. Even a single variable domain (or half of an Fv comprising only three CDRs specific

for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than an entire binding site (Painter et al. (1972) Biochem. 11:1327-1337).

This knowledge of the structure of immunoglobulin proteins has now been exploited to develop multifunctional fusion proteins comprising biosynthetic antibody binding sites and one or more other domains.

The structure of these biosynthetic proteins in the region which impart the binding properties to the protein is analogous to the Fv region of a natural antibody. It comprises at least one, and preferably two domains consisting of amino acids defining V_H and V_L-like polypeptide segments connected by a linker which together form the tertiary molecular structure responsible for affinity and specificity. Each domain comprises a set of amino acid sequences analogous to immunoglobulin CDRs held in appropriate conformation by a set of sequences analogous to the framework regions (FRs) of an Fv fragment of a natural antibody.

The term CDR, as used herein, refers to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site, or a synthetic polypeptide which mimics this function. CDRs typically are not wholly homologous to hypervariable regions of natural Fvs, but rather also may include specific amino acids or amino acid sequences which flank the hypervariable region and have heretofore been considered framework not

directly determinitive of complementarity. The term FR, as used herein, refers to amino acid sequences flanking or interposed between CDRs.

The CDR and FR polypeptide segments are designed based on sequence analysis of the Fv region of preexisting antibodies or of the DNA encoding them. In one embodiment, the amino acid sequences constituting the FR regions of the BABS are analogous to the FR sequences of a first preexisting antibody, for example, a human IgG. The amino acid sequences constituting the CDR regions are analogous to the sequences from a second, different preexisting antibody, for example, the CDRs of a murine IgG. Alternatively, the CDRs and FRs from a single preexisting antibody from, e.g., an unstable or hard to culture hybridoma, may be copied in their entirety.

Practice of the invention enables the design and biosynthesis of various reagents, all of which are characterized by a region having affinity for a preselected antigenic determinant. The binding site and other regions of the biosynthetic protein are designed with the particular planned utility of the protein in mind. Thus, if the reagent is designed for intravascular use in mammals, the FR regions may comprise amino acids similar or identical to at least a portion of the framework region amino acids of antibodies native to that mammalian species. On the other hand, the amino acids comprising the CDRs may be analogous to a portion of the amino acids from the hypervariable region (and certain flanking amino acids) of an antibody having a known affinity and specificity, e.g., a murine or rat monoclonal antibody.

Other sections of native immunoglobulin protein structure, e.g., C_H and C_L, need not be present and normally are intentionally omitted from the biosynthetic proteins. However, the proteins of the invention normally comprise additional polypeptide or protein regions defining a bioactive region, e.g., a toxin or enzyme, or a site onto which a toxin or a remotely detectable substance can be attached.

The invention thus can provide intact biosynthetic antibody binding sites analogous to V_H-V_L dimers, either non-covalently associated, disulfide bonded, or preferably linked by a polypeptide sequence to form a composite V_H-V_L or V_L-V_H polypeptide which may be essentially free of antibody constant region. The invention also provides proteins analogous to an independent V_H or V_L domain, or dimers thereof. Any of these proteins may be provided in a form linked to, for example, amino acids analogous or homologous to a bioactive molecule such as a hormone or toxin.

Connecting the independently functional regions of the protein is a spacer comprising a short amino acid sequence whose function is to separate the functional regions so that they can independently assume their active tertiary conformation. The spacer can consist of an amino acid sequence present on the end of a functional protein which sequence is not itself required for its function, and/or specific sequences engineered into the protein at the DNA level.

The spacer generally may comprise between 5 and 25 residues. Its optimal length may be determined using constructs of different spacer lengths varying, for example, by units of 5 amino acids. The specific amino acids in the spacer can vary. Cysteines should be avoided. Hydrophilic amino acids are preferred. The spacer sequence may mimic the sequence of a hinge region of an immunoglobulin. It may also be designed to assume a structure, such as a helical structure. Proteolytic cleavage sites may be designed into the spacer separating the variable region-like sequences from other pendant sequences so as to facilitate cleavage of intact BABS, free of other protein, or so as to release the bioactive protein <u>in vivo</u>.

Figures 2A-2E illustrate five examples of protein structures embodying the invention that can be produced by following the teaching disclosed herein. All are characterized by a biosynthetic polypeptide defining a binding site 3, comprising amino acid sequences comprising CDRs and FRs, often derived from different immunoglobulins, or sequences homologous to a portion of CDRs and FRs from different immunoglobulins. Figure 2A depicts a single chain construct comprising a polypeptide domain 10 having an amino acid sequence analogous to the variable region of an immunoglobulin heavy chain, bound through its carboxyl end to a polypeptide linker 12, which in turn is bound to a polypeptide domain 14 having an amino acid sequence analogous to

the variable region of an immunoglobulin light chain. Of course, the light and heavy chain domains may be in reverse order. Alternatively, the binding site may comprise two substantially homologous amino acid sequences which are both analogous to the variable region of an immunoglobulin heavy or light chain.

The linker 12 should be long enough (e.g., about 15 amino acids or about 40 A to permit the chains 10 and 14 to assume their proper conformation. The linker 12 may comprise an amino acid sequence homologous to a sequence identified as "self" by the species into which it will be introduced, if drug use is intended. For example, the linker may comprise an amino acid sequence patterned after a hinge region of an immunoglobulin. The linker preferably comprises hydrophilic amino acid sequences. It may also comprise a bioactive polypeptide such as a cell toxin which is to be targeted by the binding site, or a segment easily labelled by a radioactive reagent which is to be delivered, e.g., to the site of a tumor comprising an epitope recognized by the binding site. The linker may also include one or two built-in cleavage sites, i.e., an amino acid or amino acid sequence susceptible to attack by a site specific cleavage agent as described below. This strategy permits the V_H and V_L-like domains to be separated after expression, or the linker to be excised after folding while retaining the binding site structure in non-covalent association. The amino acids of the

linker preferably are selected from among those having relatively small, unreactive side chains. Alanine, serine, and glycine are preferred.

Generally, the design of the linker involves considerations similar to the design of the spacer, excepting that binding properties of the linked domains are seriously degraded if the linker sequence is shorter than about 20A in length, i.e., comprises less than about 10 residues. Linkers longer than the approximate 40A distance between the N terminal of a native variable region and the C-terminal of its sister chain may be used, but also potentially can diminish the BABS binding properties. Linkers comprising between 12 and 18 residues are preferred. The preferred length in specific constructs may be determined by varying linker length first by units of 5 residues, and second by units of 1-4 residues after determining the best multiple of the pentameric starting units.

Additional proteins or polypeptides may be attached to either or both the amino or carboxyl termini of the binding site to produce multifunctional proteins of the type illustrated in Figures 2B-2E. As an example, in Figure 2B, a helically coiled polypeptide structure 16 comprises a protein A fragment (FB) linked to the amino terminal end of a V_H-like domain 10 via a spacer 18. Figure 2C illustrates a bifunctional protein having an effector polypeptide 20 linked via spacer 22 to the carboxyl terminus of polypeptide 14 of binding protein segment 2. This effector polypeptide 20 may

consist of, for example, a toxin, therapeutic drug, binding protein, enzyme or enzyme fragment, site of attachment for an imaging agent (e.g., to chelate a radioactive ion such as indium), or site of selective attachment to an immobilization matrix so that the BABS can be used in affinity chromatography or solid phase binding assay. This effector alternatively may be linked to the amino terminus of polypeptide 10, although trailers are preferred. Figure 2D depicts a trifunctional protein comprising a linked pair of BABS 2 having another distinct protein domain 20 attached to the N-terminus of the first binding protein segment. Use of multiple BABS in a single protein enables production of constructs having very high selective affinity for multiepitopic sites such as cell surface proteins.

The independently functional domains are attached by a spacer 18 (Figs 2B and 2D) covalently linking the C terminus of the protein 16 or 20 to the N-terminus of the first domain 10 of the binding protein segment 2, or by a spacer 22 linking the C-terminus of the second binding domain 14 to the N-terminus of another protein (Figs. 2C and 2D). The spacer may be an amino acid sequence analogous to linker sequence 12, or it may take other forms. As noted above, the spacer's primary function is to separate the active protein regions to promote their independent bioactivity and permit each region to assume its bioactive conformation independent of interference from its neighboring structure.

Figure 2E depicts another type of reagent, comprising a BABS having only one set of three CDRs, e.g., analogous to a heavy chain variable region, which retains a measure of affinity for the antigen. Attached to the carboxyl end of the polypeptide 10 or 14 comprising the FR and CDR sequences constituting the binding site 3 through spacer 22 is effector polypeptide 20 as described above.

As is evidenced from the foregoing, the invention provides a large family of reagents comprising proteins, at least a portion of which defines a binding site patterned after the variable region of an immunoglobulin. It will be apparent that the nature of any protein fragments linked to the BABS, and used for reagents embodying the invention, are essentially unlimited, the essence of the invention being the provision, either alone or linked to other proteins, of binding sites having specificities to any antigen desired.

The clinical administration of multifunctional proteins comprising a BABS, or a BABS alone, affords a number of advantages over the use of intact natural or chimeric antibody molecules, fragments thereof, and conjugates comprising such antibodies linked chemically to a second bioactive moiety. The multifunctional proteins described herein offer fewer cleavage sites to circulating proteolytic enzymes, their functional domains are connected by peptide bonds to polypeptide linker or spacer sequences, and thus the proteins have improved stability. Because of their smaller size and efficient design, the multifunctional proteins

described herein reach their target tissue more rapidly, and are cleared more quickly from the body. They also have reduced immunogenicity. In addition, their design facilitates coupling to other moieties in drug targeting and imaging application. Such coupling may be conducted chemically after expression of the BABS to a site of attachment for the coupling product engineered into the protein at the DNA level. Active effector proteins having toxic, enzymatic, binding, modulating, cell differentiating, hormonal, or other bioactivity are expressed from a single DNA as a leader and/or trailer sequence, peptide bonded to the BABS.

Design and Manufacture

The proteins of the invention are designed at the DNA level. The chimeric or synthetic DNAs are then expressed in a suitable host system, and the expressed proteins are collected and renatured if necessary. A preferred general structure of the DNA encoding the proteins is set forth in Figure 8. As illustrated, it encodes an optimal leader sequence used to promote expression in procaryotes having a built-in cleavage site recognizable by a site specific cleavage agent, for example, an endopeptidase, used to remove the leader after expression. This is followed by DNA encoding a V_H-like domain, comprising CDRs and FRs, a linker, a V_L-like domain, again comprising CDRs and FRs, a spacer, and an effector protein. After expression,

folding, and cleavage of the leader, a bifunctional protein is produced having a binding region whose specificity is determined by the CDRs, and a peptide-linked independently functional effector region.

The ability to design the BABS of the invention depends on the ability to determine the sequence of the amino acids in the variable region of monoclonal antibodies of interest, or the DNA encoding them. Hybridoma technology enables production of cell lines secreting antibody to essentially any desired substance that produces an immune response. RNA encoding the light and heavy chains of the immunoglobulin can then be obtained from the cytoplasm of the hybridoma. The 5' end portion of the mRNA can be used to prepare cDNA for subsequent sequencing, or the amino acid sequence of the hypervariable and flanking framework regions can be determined by amino acid sequencing of the V region fragments of the H and L chains. Such sequence analysis is now conducted routinely. This knowledge, coupled with observations and deductions of the generalized structure of immunoglobulin Fvs, permits one to design synthetic genes encoding FR and CDR sequences which likely will bind the antigen. These synthetic genes are then prepared using known techniques, or using the technique disclosed below, inserted into a suitable host, and expressed, and the expressed protein is purified. Depending on the host cell, renaturation techniques may be required to attain proper conformation. The various proteins are then tested for binding ability, and one having

appropriate affinity is selected for incorporation into a reagent of the type described above. If necessary, point substitutions seeking to optimize binding may be made in the DNA using conventional casette mutagenesis or other protein engineering methodology such as is disclosed below.

Preparation of the proteins of the invention also is dependent on knowledge of the amino acid sequence (or corresponding DNA or RNA sequence) of bioactive proteins such as enzymes, toxins, growth factors, cell differentiation factors, receptors, anti-metabolites, hormones or various cytokines or lymphokines. Such sequences are reported in the literature and available through computerized data banks.

The DNA sequences of the binding site and the second protein domain are fused using conventional techniques, or assembled from synthesized oligonucleotides, and then expressed using equally conventional techniques.

The processes for manipulating, amplifying, and recombining DNA which encode amino acid sequences of interest are generally well known in the art, and therefore, not described in detail herein. Methods of identifying and isolating genes encoding antibodies of interest are well understood, and described in the patent and other literature. In general, the methods involve selecting genetic material coding for amino acids which define the proteins of interest, including the CDRs and FRs of interest, according to the genetic code.

Accordingly, the construction of DNAs encoding proteins as disclosed herein can be done using known techniques involving the use of various restriction enzymes which make sequence specific cuts in DNA to produce blunt ends or cohesive ends, DNA ligases, techniques enabling enzymatic addition of sticky ends to blunt-ended DNA, construction of synthetic DNAs by assembly of short or medium length oligonucleotides, cDNA synthesis techniques, and synthetic probes for isolating immunoglobulin or other bioactive protein genes. Various promoter sequences and other regulatory DNA sequences used in achieving expression, and various types of host cells are also known and available. Conventional transfection techniques, and equally conventional techniques for cloning and subcloning DNA are useful in the practice of this invention and known to those skilled in the art. Various types of vectors may be used such as plasmids and viruses including animal viruses and bacteriophages. The vectors may exploit various marker genes which impart to a successfully transfected cell a detectable phenotypic property that can be used to identify which of a family of clones has successfully incorporated the recombinant DNA of the vector.

One method for obtaining DNA encoding the proteins disclosed herein is by assembly of synthetic oligonucleotides produced in a conventional, automated, polynucleotide synthesizer followed by ligation with appropriate ligases. For example, overlapping, complementary DNA fragments comprising 15 bases may be synthesized semi manually using

phosphoramidite chemistry, with end segments left unphosphorylated to prevent polymerization during ligation. One end of the synthetic DNA is left with a "sticky end" corresponding to the site of action of a particular restriction endonuclease, and the other end is left with an end corresponding to the site of action of another restriction endonuclease. Alternatively, this approach can be fully automated. The DNA encoding the protein may be created by synthesizing longer single strand fragments (e.g., 50-100 nucleotides long) in, for example, a Biosearch oligonucleotide synthesizer, and then ligating the fragments.

A method of producing the BABS of the invention is to produce a synthetic DNA encoding a polypeptide comprising, e.g., human FRs, and intervening "dummy" CDRs, or amino acids having no function except to define suitably situated unique restriction sites. This synthetic DNA is then altered by DNA replacement, in which restriction and ligation is employed to insert synthetic oligonucleotides encoding CDRs defining a desired binding specificity in the proper location between the FRs. This approach facilitates empirical refinement of the binding properties of the BABS.

This technique is dependent upon the ability to cleave a DNA corresponding in structure to a variable domain gene at specific sites flanking nucleotide sequences encoding CDRs. These restriction sites in some cases may be found in the native gene. Alternatively, non-native restriction sites may be engineered into the nucleotide sequence

resulting in a synthetic gene with a different sequence of nucleotides than the native gene, but encoding the same variable region amino acids because of the degeneracy of the genetic code. The fragments resulting from endonuclease digestion, and comprising FR-encoding sequences, are then ligated to non-native CDR-encoding sequences to produce a synthetic variable domain gene with altered antigen binding specificity. Additional nucleotide sequences encoding, for example, constant region amino acids or a bioactive molecule may then be linked to the gene sequences to produce a bifunctional protein.

The expression of these synthetic DNA's can be achieved in both prokaryotic and eucaryotic systems via transfection with an appropriate vector. In E. coli and other microbial hosts, the synthetic genes can be expressed as fusion protein which is subsequently cleaved. Expression in eucaryotes can be accomplished by the transfection of DNA sequences encoding CDR and FR region amino acids and the amino acids defining a second function into a myeloma or other type of cell line. By this strategy intact hybrid antibody molecules having hybrid Fv regions and various bioactive proteins including a biosynthetic binding site may be produced. For fusion protein expressed in bacteria, subsequent proteolytic cleavage of the isolated fusions can be performed to yield free BABS, which can be renatured to obtain an intact biosynthetic, hybrid antibody binding site.

Heretofore, it has not been possible to cleave the heavy and light chain region to separate the variable and constant regions of an immunoglobulin so as to produce intact Fv, except in specific cases not of commercial utility. However, one method of producing BABS in accordance with this invention is to redesign DNAs encoding the heavy and light chains of an immunoglobulin, optionally altering its specificity or humanizing its FRs, and incorporating a cleavage site and "hinge region" between the variable and constant regions of both the heavy and light chains. Such chimeric antibodies can be produced in transfectomas or the like and subsequently cleaved using a preselected endopeptidase.

The hinge region is a sequence of amino acids which serve to promote efficient cleavage by a preselected cleavage agent at a preselected, built-in cleavage site. It is designed to promote cleavage preferentially at the cleavage site when the polypeptide is treated with the cleavage agent in an appropriate environment.

The hinge region can take many different forms. Its design involves selection of amino acid residues (and a DNA fragment encoding them) which impart to the region of the fused protein about the cleavage site an appropriate polarity, charge distribution, and stereochemistry which, in the aqueous environment where the cleavage takes place, efficiently exposes the cleavage site to the cleavage agent in preference to other potential cleavage sites that may be present in the polypeptide, and/or to

improve the kinetics of the cleavage reaction. In specific cases, the amino acids of the hinge are selected and assembled in sequence based on their known properties, and then the fused polypeptide sequence is expressed, tested, and altered for refinement.

The hinge region is free of cysteine. This enables the cleavage reaction to be conducted under conditions in which the protein assumes its tertiary conformation, and may be held in this conformation by intramolecular disulfide bonds. It has been discovered that in these conditions access of the protease to potential cleavage sites which may be present within the target protein is hindered. The hinge region may comprise an amino acid sequence which includes one or more proline residues. This allows formation of a substantially unfolded molecular segment. Aspartic acid, glutamic acid, arginine, lysine, serine, and threonine residues maximize ionic interactions and may be present in amounts and/or in sequence which renders the moiety comprising the hinge water soluble.

The cleavage site preferably is immediately adjacent the Fv polypeptide chains and comprises one amino acid or a sequence of amino acids exclusive of any sequence found in the amino acid structure of the chains in the Fv. The cleavage site preferably is designed for unique or preferential cleavage by a specific selected agent. Endopeptidases are preferred, although non-enzymatic (chemical) cleavage agents may be used. Many useful cleavage agents, for instance, cyanogen bromide, dilute acid, trypsin,

Staphylococcus aureus V-8 protease, post proline cleaving enzyme, blood coagulation Factor Xa, enterokinase, and renin, recognize and preferentially or exclusively cleave particular cleavage sites. One currently preferred cleavage agent is V-8 protease. The currently preferred cleavage site is a Glu residue. Other useful enzymes recognize multiple residues as a cleavage site, e.g., factor Xa (Ile-Glu-Gly-Arg) or enterokinase (Asp-Asp-Asp-Asp-Lys). The principles of this selective cleavage approach may also be used in the design of the linker and spacer sequences of the multifunctional constructs of the invention where an exciseable linker or selectively cleavable linker or spacer is desired.

Design of Synthetic V_H and V_L Mimics

FRs from the heavy and light chain murine anti-digoxin monoclonal 26-10 (Figures 4A and 4B) were encoded on the same DNAs with CDRs from the murine anti-lysozyme monoclonal glp-4 heavy chain (Figure 3 sequence 1) and light chain to produce V_H (Figure 4C) and V_L (Figure 4D) regions together defining a biosynthetic antibody binding site which is specific for lysozyme. Murine CDRs from both the heavy and light chains of monoclonal glp-4 were encoded on the same DNAs with FRs from the heavy and light chains of human myeloma antibody NEWM (Figures 4E and 4F). The resulting interspecies chimeric antibody binding domain has reduced immunogenicity in humans because of its human FRs, and specificity for lysozyme because of its murine CDRs.

A synthetic DNA was designed to facilitate CDR insertions into a human heavy chain FR and to facilitate empirical refinement of the resulting chimeric amino acid sequence. This DNA is depicted in Figure 5.

A synthetic, bifunctional FB-binding site protein was also designed at the DNA level, expressed, purified, renatured, and shown to bind specifically with a preselected antigen (digoxin) and Fc. The detailed primary structure of this construct is shown in Figure 6; its tertiary structure is illustrated schematically in Figure 2B.

Details of these and other experiments, and additional design principles on which the invention is based, are set forth below.

GENE DESIGN AND EXPRESSION

Given known variable region DNA sequences, synthetic V_L and V_H genes may be designed which encode native or near native FR and CDR amino acid sequences from an antibody molecule, each separated by unique restriction sites located as close to FR-CDR and CDR-FR borders as possible. Alternatively, genes may be designed which encode native FR sequences which are similar or identical to the FRs of an antibody molecule from a selected species, each separated by "dummy" CDR sequences containing strategically located restriction sites. These DNAs serve as starting materials for producing BABS, as the native or "dummy" CDR sequences may be excised and replaced with sequences encoding the CDR

amino acids defining a selected binding site. Alternatively, one may design and directly synthesize native or near-native FR sequences from a first antibody molecule, and CDR sequences from a second antibody molecule. Any one of the V_H and V_L sequences described above may be linked together directly, via an amino acids chain or linker connecting the C-terminus of one chain with the N-terminus of the other.

These genes, once synthesized, may be cloned with or without additional DNA sequences coding for, e.g., an antibody constant region, enzyme, or toxin, or a leader peptide which facilitates secretion or intracellular stability of a fusion polypeptide. The genes then can be expressed directly in an appropriate host cell, or can be further engineered before expression by the exchange of FR, CDR, or "dummy" CDR sequences with new sequences. This manipulation is facilitated by the presence of the restriction sites which have been engineered into the gene at the FR-CDR and CDR-FR borders.

Figure 3 illustrates the general approach to designing a chimeric V_H; further details of exemplary designs at the DNA level are shown in Figures 4A-4F. Figure 3, lines 1 and 2, show the amino acid sequences of the heavy chain variable region of the murine monoclonals glp-4 (anti-lysozyme) and 26-10 (anti-digoxin), including the four FR and three CDR sequences of each. Line 3 shows the sequence of a chimeric V_H which comprises 26-10 FRs and glp-4 CDRs. As illustrated, the hybrid protein of line 3 is identical to the native protein

of line 2, except that 1) the sequence TFTNYYIHWLK has replaced the sequence IFTDFYMNWVR, 2) EWIGWIYPGNGNTKYNENFKG has replaced DYIGYISPYSGVTGYNQKFKG, 3) RYTHYYF has replaced GSSGNKWAM, and 4) A has replaced V as the sixth amino acid beyond CDR-2. These changes have the effect of changing the specificity of the 26-10 V_H to mimic the specificity of glp-4. The Ala to Val single amino acid replacement within the relatively conserved framework region of 26-10 is an example of the replacement of an amino acid outside the hypervariable region made for the purpose of altering specificity by CDR replacement. Beneath sequence 3 of Figure 3, the restriction sites in the DNA encoding the chimeric V_H (see Figures 4A-4F) are shown which are disposed about the CDR-FR borders.

Lines 4 and 5 of Figure 3 represent another construct. Line 4 is the full length V_H of the human antibody NEWM. That human antibody may be made specific for lysozyme by CDR replacement as shown in line 5. Thus, for example, the segment TFTNYYIHWLK from glp-4 replaces TFSNDYYTWVR of NEWM, and its other CDRs are replaced as shown. This results in a V_H comprising a human framework with murine sequences determining specificity.

By sequencing any antibody, or obtaining the sequence from the literature, in view of this disclosure one skilled in the art can produce a BABS of any desired specificity comprising any desired framework region. Diagrams such as Figure 3 comparing the amino acid sequence are valuable in suggesting which particular amino acids should be

replaced to determine the desired complementarity. Expressed sequences may be tested for binding and refined by exchanging selected amino acids in relatively conserved regions, based on observation of trends in amino acid sequence data and/or computer modeling techniques.

Significant flexibility in V_H and V_L design is possible because the amino acid sequences are determined at the DNA level, and the manipulation of DNA can be accomplished easily.

For example, the DNA sequence for murine V_H and V_L 26-10 containing specific restriction sites flanking each of the three CDRs was designed with the aid of a commercially available computer program which performs combined reverse translation and restriction site searches ("RV.exe" by Compugene, Inc.). The known amino acid sequences for V_H and V_L 26-10 polypeptides were entered, and all potential DNA sequences which encode those peptides and all potential restriction sites were analyzed by the program. The program can, in addition, select DNA sequences encoding the peptide using only codons preferred by E. coli if this bacterium is to be host expression organism of choice. Figures 4A and 4B show an example of program output. The nucelic acid sequences of the synthetic gene and the corresponding amino acids are shown. Sites of restriction endonuclease cleavage are also indicated. The CDRs of these synthetic genes are underlined.

The DNA sequences for the synthetic 26-10 V_H and V_L are designed so that one or both of the restriction sites flanking each of the three CDRs are unique. A six base site (such as that recognized by Bsm I or BspM I) is preferred, but where six base sites are not possible, four or five base sites are used. These sites, if not already unique, are rendered unique within the gene by eliminating other occurrences within the gene without altering necessary amino acid sequences. Preferred cleavage sites are those that, once cleaved, yield fragments with sticky ends just outside of the boundary of the CDR within the framework. However, such ideal sites are only occasionally possible because the FR-CDR boundary is not an absolute one, and because the amino acid sequence of the FR may not permit a restriction site. In these cases, flanking sites in the FR which are more distant from the predicted boundary are selected.

Figure 5 discloses the nucleotide and corresponding amino acid sequence (shown in standard single letter code) of a synthetic DNA comprising a master framework gene having the generic structure:

$$R_1\text{-}FR_1\text{-}X_1\text{-}FR_2\text{-}X_2\text{-}FR_3\text{-}X_3\text{-}FR_4\text{-}R_2$$

where R_1 and R_2 are restricted ends which are to be ligated into a vector, and X_1, X_2, and X_3 are DNA sequences whose function is to provide convenient restriction sites for CDR insertion. This particular DNA has murine FR sequences and unique, 6-base restriction sites adjacent the FR borders so

that nucleotide sequences encoding CDRs from a desired monoclonal can be inserted easily. Restriction endonuclease digestion sites are indicated with their abbreviations; enzymes of choice for CDR replacement are underscored. Digestion of the gene with the following restriction endonucleases results in 3' and 5' ends which can easily be matched up with and ligated to native or synthetic CDRs of desired specificity; KpnI and BstXI are used for ligation of CDR_1; XbaI and DraI for CDR_2; and BssHII and ClaI for CDR_3.

OLIGONUCLEOTIDE SYNTHESIS

The synthetic genes and DNA fragments designed as described above preferably are produced by assembly of chemically synthesized oligonucleotides. 15-100mer oligonucleotides may be synthesized on a Biosearch DNA Model 8600 Synthesizer, and purified by polyacrylamide gel electrophoresis (PAGE) in Tris-Borate-EDTA buffer (TBE). The DNA is then electroeluted from the gel. Overlapping oligomers may be phosphorylated by T4 polynucleotide kinase and ligated into larger blocks which may also be purified by PAGE.

CLONING OF SYNTHETIC OLIGONUCLEOTIDES

The blocks or the pairs of longer oligonucleotides may be cloned into *E. coli* using a suitable, e.g., pUC, cloning vector. Initially, this vector may be altered by single strand mutagenesis to

eliminate residual six base altered sites. For example, V_H may be synthesized and cloned into pUC as five primary blocks spanning the following restriction sites: 1. EcoRI to first NarI site; 2. first NarI to XbaI; 3. XbaI to SalI; 4. SalI to NcoI; 5. NcoI to BamHI. These cloned fragments may then be isolated and assembled in several three-fragment ligations and cloning steps into the pUC8 plasmid. Desired ligations selected by PAGE are then transformed into, for example, E. coli strain JM83, and plated onto LB Ampicillin + Xgal plates according to standard procedures. The gene sequence may be confirmed by supercoil sequencing after cloning, or after subcloning into M13 via the dideoxy method of Sanger.

PRINCIPLE OF CDR EXCHANGE

Three CDRs (or alternatively, four FRs) can be replaced per V_H or V_L. In simple cases, this can be accomplished by cutting the shuttle pUC plasmid containing the respective genes at the two unique restriction sites flanking each CDR or FR, removing the excised sequence, and ligating the vector with a native nucleic acid sequence or a synthetic oligonucleotide encoding the desired CDR or FR. This three part procedure would have to be repeated three times for total CDR replacement and four times for total FR replacement. Alternatively, a synthetic nucleotide encoding two consecutive CDRs separated by the appropriate FR can be ligated to a pUC or other plasmid containing a gene whose

corresponding CDRs and FR have been cleaved out. This procedure reduces the number of steps required to perform CDR and/or FR exchange.

EXPRESSION OF PROTEINS

The engineered genes can be expressed in appropriate prokaryotic hosts such as various strains of E. coli, and in eucaryotic hosts such as Chinese hamster ovary cell, murine myeloma, and human myeloma/transfectoma cells.

For example, if the gene is to be expressed in E. coli, it may first be cloned into an expression vector. This is accomplished by positioning the engineered gene downstream from a promoter sequence such as trp or tac, and a gene coding for a leader peptide. The resulting expressed fusion protein accumulates in refractile bodies in the cytoplasm of the cells, and may be harvested after disruption of the cells by French press or sonication. The refractile bodies are solubilized, and the expressed proteins refolded and cleaved by the methods already established for many other recombinant proteins.

If the engineered gene is to be expressed in myeloma cells, the conventional expression system for immunoglobulins, it is first inserted into an expression vector containing, for example, the Ig promoter, a secretion signal, immunoglobulin enhancers, and various introns. This plasmid may also contain sequences encoding all or part of a constant region, enabling an entire part of a heavy or light chain to be expressed. The gene is

transfected into myeloma cells via established electroporation or protoplast fusion methods. Cells so transfected can express V_L or V_H fragments, V_{L2} or V_{H2} homodimers, V_L-V_H heterodimers, V_H-V_L or V_L-V_H single chain polypeptides, complete heavy or light immunoglobulin chains, or portions thereof, each of which may be attached in the various ways discussed above to a protein region having another function (e.g., cytotoxicity).

Vectors containing a heavy chain V region (or V and C regions) can be cotransfected with analogous vectors carrying a light chain V region (or V and C regions), allowing for the expression of noncovalently associated binding sites (or complete antibody molecules).

In the examples which follow, a specific example of how to make a single chain binding site is disclosed, together with methods employed to assess its binding properties. Thereafter, a protein construct having two functional domains is disclosed. Lastly, there is disclosed a series of additional targeted proteins which exemplify the invention.

I EXAMPLE OF CDR EXCHANGE AND EXPRESSION

The synthetic gene coding for murine V_H and V_L 26-10 shown in Figures 4A and 4B were designed from the known amino acid sequence of the protein with the aid of Compugene, a software program. These genes, although coding for the native amino acid sequences, also contain non-native and

often unique restriction sites flanking nucleic acid sequences encoding CDR's to facilitate CDR replacement as noted above.

Both the 3' and 5' ends of the large synthetic oligomers were designed to include 6-base restriction sites, present in the genes and the pUC vector. Furthermore, those restriction sites in the synthetic genes which were only suited for assembly but not for cloning the pUC were extended by "helper" cloning sites with matching sites in pUC.

Cloning of the synthetic DNA and later assembly of the gene is facilitated by the spacing of unique restriction sites along the gene. This allows corrections and modifications by cassette mutagenesis at any location. Among them are alterations near the 5' or 3' ends of the gene as needed for the adaptation to different expression vectors. For example, a PstI site is positioned near the 5' end of the V_H gene. Synthetic linkers can be attached easily between this site and a restriction site in the expression plasmid. These genes were synthesized by assembling oligonucleotides as described above using a Biosearch Model 8600 DNA Synthesizer. They were ligated to vector pUC8 for transformation of _E. coli_.

Specific CDRs may be cleaved from the synthetic V_H gene by digestion with the following pairs of restriction endonucleases: HpHI and BstXI for CDR_1; XbaI and DraI for CDR_2; and BanII and BanI for CDR_3. After removal on one CDR, another CDR of desired specificity may be ligated directly

into the restricted gene, in its place if the 3' and 5' ends of the restricted gene and the new CDR contain complementary single stranded DNA sequences.

In the present example, the three CDRs of each of murine V_H 26-10 and V_L 26-10 were replaced with the corresponding CDRs of glp-4. The nucleic acid sequences and corresponding amino acid sequences of the chimeric V_H and V_L genes encoding the FRs of 26-10 and CDRs of glp-4 are shown in Figures 4C and 4D. The positions of the restriction endonuclease cleavage sites are noted with their standard abbreviations. CDR sequences are underlined as are the restriction endonucleases of choice useful for further CDR replacement.

These genes were cloned into pUC8, a shuttle plasmid. To retain unique restriction sites after cloning, the V_H-like gene was spliced into the EcoR1 and HindIII or BamHI sites of the plasmid.

Direct expression of the genes may be achieved in *E. coli*. Alternatively, the gene may be preceded by a leader sequence and expressed in *E. coli* as a fusion product by splicing the fusion gene into the host gene whose expression is regulated by interaction of a repressor with the respective operator. The protein can be induced by starvation in minimal medium and by chemical inducers. The V_H-V_L biosynthetic 26-10 gene has been expressed as such a fusion protein behind the trp and tac promoters. The gene translation product of interest may then be cleaved from the leader in the fusion protein by e.g., cyanogen bromide degradation, tryptic digestion, mild acid cleavage, and/or

digestion with factor Xa protease. Therefore, a shuttle plasmid containing a synthetic gene encoding a leader peptide having a site for mild acid cleavage, and into which has been spliced the synthetic BABS gene was used for this purpose. In addition, synthetic DNA sequences encoding a signal peptide for secretion of the processed target protein into the periplasm of the host cell can also be incorporated into the plasmid.

After harvesting the gene product and optionally releasing it from a fusion peptide, its activity as an antibody binding site and its specificity for glp-4 (lysozyme) epitope are assayed by established immunological techniques, e.g., affinity chromatography and radioimmunoassay. Correct folding of the protein to yield the proper three-dimensional conformation of the antibody binding site is prerequisite for its activity. This occurs spontaneously in a host such as a myeloma cell which naturally expresses immunoglobulin proteins. Alternatively, for bacterial expression, the protein forms inclusion bodies which, after harvesting, must be subjected to a specific sequence of solvent conditions (e.g., diluted 20 X from 8 M urea 0.1 M Tris-HCl pH 9 into 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4 (Hochman et al. (1976) Biochem. 15:2706-2710) to assume its correct conformation and hence its active form.

Figures 4E and 4F show the DNA and amino acid sequence of chimeric V_H and V_L comprising human FRs from NEWM and murine CDRs from glp-4. The

CDRs are underlined, as are restriction sites of choice for further CDR replacement or empirically determined refinement.

These constructs also constitute master framework genes, this time constructed of human framework sequences. They may be used to construct BABS of any desired specificity by appropriate CDR replacement.

Binding sites with other specificities have also been designed using the methodologies disclosed herein. Examples include those having FRs from the human NEWM antibody and CDRs from murine 26-10 (Figure 9A), murine 26-10 FRs and G-loop CDRs (Figure 9B), FRs and CDRs from murine MOPC-315 (Figure 9C), FRs and CDRs from an anti-human carcinoembryonic antigen monoclonal antibody (Figure 9D), and FRs and CDRs 1, 2, and 3 from V_L and FRs and CDR 1 and 3 from the V_H of the anti-CEA antibody, with CDR 2 from a consensus immunoglobulin gene (Figure 9E).

II. Model Binding Site:

The digoxin binding site of the $IgG_{2a,k}$ monoclonal antibody 26-10 has been analyzed by Mudgett-Hunter and colleagues (unpublished). The 26-10 V region sequences were determined from both amino acid sequencing and DNA sequencing of 26-10 H and L chain mRNA transcripts (D. Panka, J.N. & M.N.M., unpublished data). The 26-10 antibody exhibits a high digoxin binding affinity [$K_o = 5.4 \times 10^9 M^{-1}$] and has a well-defined specificity profile, providing a baseline for comparison with the biosynthetic binding sites mimicking its structure.

Protein Design:

Crystallographically determined atomic coordinates for Fab fragments of 26-10 were obtained from the Brookhaven Data Bank. Inspection of the available three-dimensional structures of Fv regions within their parent Fab fragments indicated that the Euclidean distance between the C-terminus of the V_H domain and the N-terminus of the V_L domain is about 35 A. Considering that the peptide unit length is approximately 3.8 A, a 15 residue linker was selected to bridge this gap. The linker was designed so as to exhibit little propensity for secondary structure and not to interfere with domain folding. Thus, the 15 residue sequence $(Gly-Gly-Gly-Gly-Ser)_3$ was selected to connect the V_H carboxyl- and V_L amino-termini.

Binding studies with single chain binding sites having less than or greater than 15 residues demonstrate the importance of the prerequisite distance which must separate V_H from V_L; for example, a $(Gly_4-Ser)_1$ linker does not demonstrate binding activity, and those with $(Gly_4-Ser)_5$ linkers exhibit very low activity compared to those with $(Gly_4-Ser)_3$ linkers.

Gene Synthesis:

Design of the 744 base sequence for the synthetic binding site gene was derived from the Fv protein sequence of 26-10 by choosing codons

frequently used in *E. coli*. The model of this representative synthetic gene is shown in Figure 8, discussed previously. Synthetic genes coding for the *trp* promoter-operator, the modified *trp* LE leader peptide (MLE), the sequence of which is shown in Figure 10A, and V_H were prepared largely as described previously. The gene coding for V_H was assembled from 46 chemically synthesized oligonucleotides, all 15 bases long, except for terminal fragments (13 to 19 bases) that included cohesive cloning ends. Between 8 and 15 overlapping oligonucleotides were enzymatically ligated into double stranded DNA, cut at restriction sites suitable for cloning (NarI, XbaI, SalI, SacII, SacI), purified by PAGE on 8% gels, and cloned in pUC which was modified to contain additional cloning sites in the polylinker. The cloned segments were assembled stepwise into the complete gene mimicking V_H by ligations in the pUC cloning vector.

The gene mimicking 26-10 V_L was assembled from 12 long synthetic polynucleotides ranging in size from 33 to 88 base pairs, prepared in automated DNA synthesizers (Model 6500, Biosearch, San Rafael, CA; Model 380A, Applied Biosystems, Foster City, CA). Five individual double stranded segments were made out of pairs of long synthetic oligonucleotides spanning six-base restriction sites in the gene (AatII, BstEII, PpnI, HindIII, BglII, and PstI). In one case, four long overlapping strands were combined and cloned. Gene fragments bounded by restriction sites for assembly that were absent from the pUC polylinker, such as AatII and BstEII, were flanked by EcoRI and BamHI ends to facilitate cloning.

The linker between V_H and V_L, encoding (Gly-Gly-Gly-Gly-Ser)$_3$, was cloned from two long synthetic oligonucleotides, 54 and 62 bases long, spanning SacI and AatII sites, the latter followed by an EcoRI cloning end. The complete single chain binding site gene was assembled from the V_H, V_L, and linker genes to produce a construct, corresponding to aspartyl-prolyl-V_H-<linker>-V_L, flanked by EcoRI and PstI restriction sites.

The trp promoter-operator, starting from its SspI site, was assembled from 12 overlapping 15 base oligomers, and the MLE leader gene was assembled from 24 overlapping 15 base oligomers. These were cloned and assembled in pUC using the strategy of assembly sites flanked by cloning sites. The final expression plasmid was constructed in the pBR322 vector by a 3-part ligation using the sites SspI, EcoRI, and PstI (see Figure 10B). Intermediate DNA fragments and assembled genes were sequenced by the dideoxy method.

Fusion Protein Expression:

Single-chain protein was expressed as a fusion protein. The MLE leader gene (Fig. 10A) was derived from *E. coli* trp LE sequence and expressed under the control of a synthetic trp promoter and operator. *E. coli* strain JM83 was transformed with the expression plasmid and protein expression was induced in M9 minimal medium by addition of indoleacrylic acid (10 µg/ml) at a cell density with $A_{600} = 1$. The high expression levels of the

fusion protein resulted in its accumulation as insoluble protein granules, which were harvested from cell paste (Figure 11, Lane 1).

Fusion Protein Cleavage:

The MLE leader was removed from the binding site protein by acid cleavage of the Asp-Pro peptide bond engineered at the junction of the MLE and binding site sequences. The washed protein granules containing the fusion protein were cleaved in 6 M guanidine-HCl + 10% acetic acid, pH 2.5, incubated at 37°C for 96 hrs. The reaction was stopped through precipitation by addition of a 10-fold excess of ethanol with overnight incubation at -20°C, followed by centrifugation and storage at -20°C until further purification (Figure 11, Lane 2).

Protein Purification:

The acid cleaved binding site was separated from remaining intact fused protein species by chromatography on DEAE cellulose. The precipitate obtained from the cleavage mixture was redissolved in 6 M guanidine-HCl + 0.2 M Tris-HCl, pH 8.2, + 0.1 M 2-mercaptoethanol and dialyzed exhaustively against 6 M urea + 2.5 mM Tris-HCl, pH 7.5, + 1 mM EDTA. 2-Mercaptoethanol was added to a final concentration of 0.1 M, the solution was incubated for 2 hrs at room temperature and loaded onto a 2.5 X 45 cm column of DEAE cellulose (Whatman DE 52), equilibrated with

6 M urea + 2.5 mM Tris-HCl + 1 mM EDTA, pH 7.5. The intact fusion protein bound weakly to the DE 52 column such that its elution was retarded relative to that of the binding protein. The first protein fractions which eluted from the column after loading and washing with urea buffer contained BABS protein devoid of intact fusion protein. Later fractions contaminated with some fused protein were pooled, rechromatographed on DE 52, and recovered single chain binding protein combined with other purified protein into a single pool (Figure 11, Lane 3).

Refolding:

The 26-10 binding site mimic was refolded as follows: the DE 52 pool, disposed in 6 M urea + 2.5 mM Tris-HCl + 1 mM EDTA, was adjusted to pH 8 and reduced with 0.1 M 2-mercaptoethanol at 37°C for 90 min. This was diluted at least 100-fold with 0.01 M sodium acetate, pH 5.5, to a concentration below 10 µg/ml and dialyzed at 4°C for 2 days against acetate buffer.

Affinity Chromatography:

Purification of active binding protein by affinity chromatography at 4°C on a ouabain-amine-Sepharose column was performed. The dilute solution of refolded protein was loaded directly onto a pair of tandem columns, each containing 3 ml of resin equilibrated with the 0.01 M acetate buffer, pH 5.5. The columns were washed

individually with an excess of the acetate buffer, and then by sequential additions of 5 ml each of 1 M NaCl, 20 mM ouabain, and 3 M potassium thiocyanate dissolved in the acetate buffer, interspersed with acetate buffer washes. Since digoxin binding activity was still present in the eluate, the eluate was pooled and concentrated 20-fold by ultrafiltration (PM 10 membrane, 200 ml concentrator; Amicon), reapplied to the affinity columns, and eluted as described. Fractions with significant absorbance at 280 nm were pooled and dialyzed against PBSA or the above acetate buffer. The amounts of protein in the DE 52 and ouabain-Sepharose pools were quantitated by amino acid analysis following dialysis against 0.01 M acetate buffer. The results are shown below in Table 1.

TABLE 1

Estimated Yields of BABS Protein During Purification

Step	Wet wt. Per l	mg protein	Cleavage yield (%) prior step	Yield relative to fusion protein
Cell paste	12.0 g	1440.0 mg[a]		
Fusion protein Granules	2.3 g	480.0 mg[a,b]	100.0%	100.0%
Acid Cleavage/ DE 52 pool		144.0 mg	38.0[e]	38.0[e]
Ouabain-Sepharose pool		18.1 mg	12.6[d]	4.7[e]

[a]Determined by Lowry protein analysis

[b]Determined by absorbance measurements

[c]Determined by amino acid analysis

[d]Calculated from the amount of BABS protein specifically eluted from ouabain-Sepharose relative to that applied to the resin; values were determined by amino acid analysis

[e]Percentage yield calculated on a molar basis

Sequence Analysis of Gene and Protein:

The complete gene was sequenced in both directions using the dideoxy method of Sanger which confirmed the gene was correctly assembled. The protein sequence was also verified by protein sequencing. Automated Edman degradation was conducted on intact protein (residues 1-40), as well as on two major CNBr fragments (residues 108-129 and 140-159) with a Model 470A gas phase sequencer equipped with a Model 120A on-line phenylthiohydantoin-amino acid analyzer (Applied Biosystems, Foster City, CA). Homogeneous binding protein fractionated by SDS-PAGE and eluted from gel strips with water, was treated with a 20,000-fold excess of CNBr, in 1% trifluoroacetic acid-acetonitrile (1:1), for 12 hrs at 25° (in the dark). The resulting fragments were separated by SDS-PAGE and transferred electrophoretically onto an Immobilon membrane (Millipore, Bedford, MA), from which stained bands were cut out and sequenced.

Specificity Determination:

Specificities of anti-digoxin 26-10 Fab and the BABS were assessed by radioimmunoassay. Wells of microtiter plates were coated with affinity-purified goat anti-murine Fab fragment (ICN ImmunoBiologicals, Lisle, IL) at 10 μg/ml in PBSA overnight at 4°C. After the plates were washed and blocked with 1% horse serum in PBSA, solutions (50 μl) containing 26-10 Fab or the BABS in either PBSA or 0.01 M sodium acetate at pH 5.5 were added to the wells and

incubated 2-3 hrs at room temperature. After unbound antibody fragment was washed from the wells, 25 µl of a series of concentrations of cardiac glycosides (10^{-4} to 10^{-11} M in PBSA) were added. The cardiac glycosides tested included digoxin, digitoxin, digoxigenin, digitoxigenin, gitoxin, ouabain, and acetyl strophanthidin. After the addition of ^{125}I-digoxin (25 µl, 50,000 cpm; Cambridge Diagnostics, Billerica, MA) to each well, the plates were incubated overnight at 4°C, washed and counted. The inhibition curves are plotted in Figure 12. The relative affinities for each digoxin analogue were calculated by dividing the concentration of each analogue at 50% inhibition by the concentration of digoxin (or digoxigenin) that gave 50% inhibition. There is a displacement of inhibition curves for the BABS to lower glycoside concentrations than observed for 26-10 Fab, because less active BABS than 26-10 Fab was bound to the plate. When 0.25 M urea was added to the BABS in 0.01 M sodium acetate, pH 5.5, more active sFv was bound to the goat anti-murine Fab coating on the plate. This caused the BABS inhibition curves to shift toward higher glycoside concentrations, closer to the position of those for 26-10 Fab, although maintaining the relative positions of curves for sFv obtained in acetate buffer alone. The results, expressed as normalized concentration of inhibitor giving 50% inhibition of ^{125}I-digoxin binding, are shown in Table 2.

TABLE 2

26-10 Antibody Species	Normalizing Glycoside	D	DG	DO	DOG	A-S	G	O
Fab	Digoxin	1.0	1.2	0.9	1.0	1.3	9.6	15
	Digoxigenin	0.9	1.0	0.8	0.9	1.1	8.1	13
BABS	Digoxin	1.0	7.3	2.0	2.6	5.9	62	150
	Digoxigenin	0.1	1.0	0.3	0.4	0.8	8.5	21

D = Digoxin

DG = Digoxigenin

DO = Digitoxin

DOG = Digitoxigenin

A-S = Acetyl Strophanthidin

G = Gitoxin

O = Ouabain

Affinity Determination:

Association constants were measured by equilibrium binding studies. In immunoprecipitation experiments, 100 µl of ^{3}H-digoxin (New England Nuclear, Billerica, MA) at a series of concentrations (10^{-7} M to 10^{-11} M) were added to 100 µl of 26-10 Fab or the BABS at a fixed concentration. After 2-3 hrs of incubation at room temperature, the protein was precipitated by the addition of 100 µl goat antiserum to murine Fab fragment (ICN Immuno-

Biologicals), 50 µl of the IgG fraction of rabbit anti-goat IgG (ICN ImmunoBiologicals), and 50 µl of a 10% suspension of protein A-Sepharose (Sigma). Following 2 hrs at 4°C, bound and free antigen were separated by vacuum filtration on glass fiber filters (Vacuum Filtration Manifold, Millipore, Bedford, MA). Filter disks were then counted in 5 ml of scintillation fluid with a Model 1500 Tri-Carb Liquid Scintillation Analyzer (Packard, Sterling, VA). The association constants, K_o, were calculated from Scatchard analyses of the untransformed radioligand binding data using LIGAND, a non-linear curve fitting program based on mass action. K_os were also calculated by Sips plots and binding isotherms shown in Figure 13A for the BABS and 13B for the Fab. For binding isotherms, data are plotted as the concentration of digoxin bound versus the log of the unbound digoxin concentration, and the dissociation constant is estimated from the ligand concentration at 50% saturation. These binding data are also plotted in linear form as Sips plots (inset), having the same abscissa as the binding isotherm but with the ordinate representing log r/(n-r), defined below. The average intrinsic association constant (K_o) was calculated from the modified Sips equation (39), $\log (r/n-r) = a \log C - a \log K_o$, where r equals moles of digoxin bound per mole of antibody at an unbound digoxin concentration equal to C; n is the number of moles of digoxin bound at saturation of the antibody binding site, and a is an index of heterogeneity which describes the distribution of association constants about the average intrinsic

association constant K_o. Least squares linear regression analysis of the data indicated correlation coefficients for the lines obtained were 0.96 for the BABS and 0.99 for 26-10 Fab. A summary of the calculated association constants are shown below in Table 3.

TABLE 3

Method of Data Analysis	Association Constant, K_o	
	K_o (BABS), M^{-1}	K_o (Fab), M^{-1}
Scatchard plot	$(3.2 \pm 0.9) \times 10^7$	$(1.9 \pm 0.2) \times 10^8$
Sips plot	2.6×10^7	1.8×10^8
Binding isotherm	5.2×10^7	3.3×10^8

III. Synthesis of a Multifunctional Protein

A nucleic acid sequence encoding the single chain binding site described above was fused with a sequence encoding the FB fragment of protein A as a leader to function as a second active region. As a spacer, the native amino acids comprising the last 11 amino acids of the FB fragment bonded to an Asp-Pro dilute acid cleavage site was employed. The FB binding domain of the FB consists of the immediately preceding 43 amino acids which assume a helical configuration (see Fig. 2B).

The gene fragments are synthesized using a Biosearch DNA Model 8600 Synthesizer as described above. Synthetic oligonucleotides are cloned according to established protocol described above using the pUC8 vector transfected into E. coli. The completed fused gene set forth in Figure 6A is then expressed in E. coli.

After sonication, inclusion bodies were collected by centrifugation, and dissolved in 6 M guanidine hydrochloride (GuHCl), 0.2 M Tris, and 0.1 M 2-mercaptoethanol (BME), pH 8.2. The protein was denatured and reduced in the solvent overnight at room temperature. Size exclusion chromatography was used to purify fusion protein from the inclusion bodies. A Sepharose 4B column (1.5 X 80 cm) was run in a solvent of 6 M GuHCl and 0.01 M NaOAc, pH 4.75. The protein solution was applied to the column at room temperature in 0.5-1.0 ml amounts. Fractions were collected and precipitated with cold ethanol. These were run on SDS gels, and fractions rich in the recombinant protein (approximately 34,000 D) were pooled. This offers a simple first step for cleaning up inclusion body preparations without suffering significant proteolytic degradation.

For refolding, the protein was dialyzed against 100 ml of the same GuHCl-Tris-BME solution, and dialysate was diluted 11-fold over two days to 0.55 M GuHCl, 0.01 M Tris, and 0.01 M BME. The dialysis sacks were then transferred to 0.01 M NaCl, and the protein was dialyzed exhaustively before being assayed by RIA's for binding of ^{125}I-labelled digoxin. The refolding procedure can be simplified by

making a rapid dilution with water to reduce the GuHCl concentration to 1.1 M, and then dialyzing against phosphate buffered saline (0.15 M NaCl, 0.05 M potassium phosphate, pH 7, containing 0.03% NaN_3), so that it is free of any GuHCl within 12 hours. Product of both types of preparation showed binding activity, as indicated in Figure 7A.

Demonstration of Bifunctionality:

This protein with an FB leader and a fused BABS is bifunctional; the BABS can bind the antigen and the FB can bind the Fc regions of immunoglobulins. To demonstrate this dual and simulataneous activity several radioimmunoassays were performed.

Properties of the binding site were probed by a modification of an assay developed by Mudgett-Hunter et al. (J. Immunol. (1982) 129:1165-1172; Molec. Immunol. (1985) 22:477-488), so that it could be run on microtiter plates as a solid phase sandwich assay. Binding data were collected using goat anti-murine Fab antisera (gAmFab) as the primary antibody that initially coats the wells of the plate. These are polyclonal antisera which recognize epitopes that appear to reside mostly on framework regions. The samples of interest are next added to the coated wells and incubated with the gAmFab, which binds species that exhibit appropriate antigenic sites. After washing away unbound protein, the wells are exposed to ^{125}I-labelled (radioiodinated) digoxin conjugates, either as ^{125}I-dig-BSA or ^{125}I-dig-lysine.

The data are plotted in Figure 7A, which shows the results of a dilution curve experiment in which the parent 26-10 antibody was included as a control. The sites were probed with ^{125}I-dig-BSA as described above, with a series of dilutions prepared from initial stock solutions, including both the slowly refolded (1) and fast diluted/quickly refolded (2) single chain proteins. The parallelism between all three dilution curves indicates that gAmFab binding regions on the BABS molecule are essentially the same as on the Fv of authentic 26-10 antibody, i.e., the surface epitopes appear to be the same for both proteins.

The sensitivity of these assays is such that binding affinity of the Fv for digoxin must be at least 10^6. Experimental data on digoxin binding yielded binding constants in the range of 10^8 to 10^9 M^{-1}. The parent 26-10 antibody has an affinity of 5.4 X 10^9 M^{-1}. Inhibition assays also indicate the binding of ^{125}I-dig-lysine, and can be inhibited by unlabelled digoxin, digoxigenin, digitoxin, digitoxigenin, gitoxin, acetyl strophanthidin, and ouabain in a way largely parallel to the parent 26-10 Fab. This indicates that the specificity of the biosynthetic protein is substantially identical to the original monoclonal.

In a second type of assay, Digoxin-BSA is used to coat microtiter plates. Renatured BABS (FB-BABS) is added to the coated plates so that only molecules that have a competent binding site can stick to the plate. ^{125}I-labelled rabbit IgG (radioligand) is mixed with bound FB-BABS on the

plates. Bound radioactivity reflects the interation of IgG with the FB domain of the BABS, and the specificity of this binding is demonstrated by its inhibition with increasing amounts of FB, Protein A, rabbit IgG, IgG2a, and IgG1, as shown in Figure 7B.

The following species were tested in order to demonstrate authentic binding: unlabelled rabbit IgG and IgG2a monoclonal antibody (which binds competiviely to the FB domain of the BABS); and protein A and FB (which bind competively to the radioligand). As shown in Figure 7B, these species are found to completely inhibit radioligand binding, as expected. A monoclonal antibody of the IgG1 subclass binds poorly to the FB, as expected, inhibiting only about 34% of the radioligand from binding. These data indicate that the BABS domain and the FB domain have independent activity.

IV. <u>OTHER CONSTRUCTS</u>

Other BABS-containing protein constructed according to the invention expressible in <u>E</u>. <u>coli</u> and other host cells as described above are set forth in the drawing. These proteins may be bifunctional or multifunctional. Each construct includes a single chain BABS linked via a spacer sequence to an effector molecule comprising amino acids encoding a biologically active effector protein such as an enzyme, receptor, toxin, or growth factor. Some examples of such constructs shown in the drawing include proteins comprising epidermal growth factor (EGF) (Figure 15A), streptavidin (Figure 15B), tumor

necrosis factor (TNF) (Figure 15C), calmodulin (Figure 15D) the beta chain of platelet derived growth factor (B-PDGF) (15E) ricin A (15F), interleukin 2 (15G) and FB dimer (15H). Each is used as a trailer and is connected to a preselected BABS via a spacer (Gly-Ser-Gly) encoded by DNA defining a BamHI restriction site. Additional amino acids may be added to the spacer for empirical refinement of the construct if necessary by opening up the Bam HI site and inserting an oligonucleotide of a desired length having BamHI sticky ends. Each gene also terminates with a PstI site to facilitate insertion into a suitable expression vector.

The BABS of the EGF and PDGF constructs may be, for example, specific for fibrin so that the EGF or PDGF is delivered to the site of a wound. The BABS for TNF and ricin A may be specific to a tumor antigen, e.g., CEA, to produce a construct useful in cancer therapy. The calmodulin construct binds radioactive ions and other metal ions. Its BABS may be specific, for example, to fibrin or a tumor antigen, so that it can be used as an imaging agent to locate a thrombus or tumor. The streptavadin construct binds with biotin with very high affinity. The biotin may be labeled with a remotely detectable ion for imaging purposes. Alternatively, the biotin may be immobilized on an affinity matrix or solid support. The BABS-streptavidin protein could then be bound to the matrix or support for affinity chromatography or solid phase immunoassay. The interleukin-2 construct could be linked, for example, to a BABS specific for a T-cell surface antigen. The

FB-FB dimer binds to Fc, and could be used with a BABS in an immunoassay or affinity purification procedure linked to a solid phase through immobilized immunoglobulin.

Figure 14 exemplifies a multifunctional protein having an effector segment as a leader. It comprises an FB-FB dimer linked through its C-terminal via an Asp-Pro dipeptide to a BABS of choice. It functions in a way very similar to the construct of Fig. 15H. The dimer binds avidly to the Fc portion of immunoglobulin. This type of construct can accordingly also be used in affinity chromatography, solid phase immunoassay, and in therapeutic contexts where coupling of immunoglobulins to another epitope is desired.

In view of the foregoing, it should be apparent that the invention is unlimited with respect to the specific types of BABS and effector proteins to be linked. Accordingly, other embodiments are within the following claims.

What is claimed is:

Claims

1. A single chain multi-functional biosynthetic protein expressed from a single gene derived by recombinant DNA techniques, said protein comprising:

a biosynthetic antibody binding site capable of binding to a preselected antigenic determinant and comprising at least one protein domain, the amino acid sequence of said domain being homologous to at least a portion of the sequence of a variable region of an immunoglobulin molecule capable of binding said preselected antigenic determinant; and, peptide bonded thereto,

a polypeptide selected from the group consisting of effector proteins having a conformation suitable for biological activity in mammals, amino acid sequences capable of sequestering an ion, and amino acid sequences capable of selective binding to a solid support.

2. The protein of claim 1 wherein said binding site comprises at least two domains connected by peptide bonds to a polypeptide linker.

3. The protein of claim 2 wherein said two domains mimic a V_H and a V_L from a natural immunoglobulin.

4. The protein of claim 2 wherein the amino acid sequence of each of said domains comprises a set of CDRs interposed between a set of FRs, each of which is respectively homologous with at least a portion of CDRs and FRs from a said variable region of an immunoglobulin molecule capable of binding said preselected antigenic determinant.

5. The protein of claim 4 wherein at least one of said domains comprises a said set of CDRs homologous to a portion of the CDRs in a first immunoglobulin and a set of FRs homologous to a portion of the FRs in a second, distinct immunoglobulin.

6. The protein of claim 2 wherein said polypeptide linker spans a distance of at least 40 angstroms is hydrophilic.

7. The protein of claim 2 wherein said polypeptide linker comprises amino acids which together assume an unstructured polypeptide configuration in aqueous solution.

8. The protein of claim 2 wherein said polypeptide linker is cysteine-free.

9. The protein of claim 2 wherein said polypeptide linker comprises a plurality of glycine or alanine residues.

10. The protein of claim 2 wherein said polypeptide linker comprises plural consecutive copies of an amino acid sequence.

11. The protein of claim 2 wherein said polypeptide linker comprises one or a pair of amino acid sequences recognizable by a site specific cleavage agent.

12. The protein of claim 4 wherein said antibody binding site binds with said antigenic determinant with a specificity at least substantially identical to the binding specificity of said immunoglobulin molecule.

13. The protein of claim 4 wherein said antibody binding site binds said antigenic determinant with an affinity of at least about 10^6 M^{-1}.

14. The protein of claim 4 wherein said antibody binding site binds said antigenic determinant with an affinity no less than about two orders of magnitude less than the binding affinity of said immunoglobulin molecule.

15. The protein of claim 1 further comprising a polypeptide spacer incorporated therein interposed between said antibody binding site and said polypeptide.

16. The protein of claim 15 wherein said polypeptide spacer comprises amino acids selectively susceptible to cleavage.

17. The protein of claim 15 wherein said spacer is hydrophilic.

18. The protein of claim 15 wherein said spacer comprises amino acids which together assume an unstructured polypeptide configuration in aqueous solution.

19. The protein of claim 15 wherein said spacer is cysteine-free.

20. The protein of claim 15 wherein said spacer comprises a plurality of glycine or alanine residues.

21. The protein of claim 15 wherein said spacer comprises plural consecutive copies of an amino acid sequence.

22. The protein of claim 1 wherein said effector protein is an enzyme, toxin, receptor, binding site, biosynthetic antibody binding site, growth factor, cell-differentiation factor, lymphokine, cytokine, hormone, or anti-metabolite.

23. The protein of claim 1 wherein said sequence capable of sequestering an ion is calmodulin, metallothionein, a fragment thereof, or an amino acid sequence rich in at least one of glutamic acid, aspartic acid, lysine, and arginine.

24. The protein of claim 1 wherein said polypeptide sequence capable of selective binding to a solid support is a positively or negatively charged amino acid sequence, a cysteine-containing amino acid sequence, streptavidin, or a fragment of protein A.

25. The protein of claim 1 comprising a plurality of biosynthetic antibody binding sites.

26. The protein of claim 1 comprising an additional biofunctional domain.

27. A DNA encoding the protein of claim 1.

28. A host cell harboring and capable of expressing the DNA of claim 27.

29. A biosynthetic binding protein expressed from DNA derived by recombinant techniques

said binding protein comprising a single polypeptide chain comprising at least two polypeptide domains connected by a polypeptide linker, the amino acid sequence of each of said polypeptide domains comprising a set of CDRs interposed between a set of FRs, each of which is respectively homologous with at least a portion of CDRs and FRs from an immunoglobulin molecule,

at least one of said domains comprising a said set of CDR amino acid sequences homologous to a portion of the CDR amino acid sequences of a first immunoglobulin molecule, and a set of FR amino acid sequences homologous to a portion of the FR sequences of a second, distinct immunoglobulin molecule,

said polypeptide domains together defining a hybrid synthetic binding site having specificity for a preselected antigen.

30. The binding protein of claim 29 wherein said domains comprise FRs homologous to a portion of the FRs of a human immunoglobulin.

31. The binding protein of claim 29 wherein said polypeptide domains are peptide bonded to a biologically active amino acid sequence.

32. The binding protein of claim 29 further comprising a radioactive atom bound to said binding protein.

33. A DNA encoding the binding protein of claim 32.

34. A host cell harboring and capable of expressing the DNA of claim 33.

35. A biosynthetic binding protein expressed from DNA derived by recombinant techniques

said binding protein comprising a single polypeptide chain comprising at least two polypeptide domains connected by a polypeptide linker, the amino acid sequence of each of said polypeptide domains comprising a set of CDRs interposed between a set of FRs, each of which is respectively homologous with at least a portion of CDRs and FRs from an immunoglobulin molecule,

said polypeptide linker comprising plural, peptide-bonded amino acids defining a polypeptide of a length sufficient to span the distance between the C-terminal end of one of said domains and the N-terminal end of the other of said domains when said binding protein assumes a conformation suitable for binding, and comprising hydrophilic amino acids which together assume an unstructured polypeptide configuration in aqueous solution,

said binding protein being capable of binding to a preselected antigenic site, determined by the collective tertiary structure of said sets of CDRs held in proper conformation by said sets of FRs and said linker when disposed in aqueous solution.

36. The binding protein of claim 35 wherein said polypeptide linker spans a distance of at least about 40A when said binding protein is disposed in aqueous solution in a conformation suitable for binding said preselected antigen.

37. The binding protein of claim 35 wherein said polypeptide linker comprises a plurality of glycine or alanine residues.

38. The binding protein of claim 35 wherein said linker comprises plural consecutive copies of an amino acid sequence.

39. The binding protein of claim 35 wherein said linker comprises $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$.

40. The binding protein of claim 35 wherein at least one of said domains comprises a said set of CDRs homologous to a portion of the CDRs in a first immunoglobulin and a set of FRs homologous to a portion of the FRs of a second, distinct, human immunoglobulin.

41. The binding protein of claim 35 wherein at least one of said polypeptide domains is peptide bonded to a biologically active amino acid sequence.

42. The binding protein of claim 35 further comprising a radioactive atom bound to said polypeptide chain.

43. A biosynthetic binding protein expressed from DNA derived by recombinant techniques,

said binding protein comprising a single polypeptide chain comprising at least two polypeptide domains connected by a polypeptide linker, the amino acid sequence of each of said polypeptide domains comprising a set of CDRs interposed between a set of FRs, each of which are respectively homologous with at least a portion of CDRs and FRs from an immunoglobulin molecule,

said binding protein being capable of binding to a preselected antigenic determinant, determined by the collective tertiary structure of said sets of CDRs held in proper conformation by said sets of FRs when disposed in aqueous solution, with a binding specificity at least substantially identical to the binding specificity of said immunoglobulin molecule comprising said homologous CDRs.

44. A biosynthetic binding protein expressed from DNA derived by recombinant techniques,

said binding protein comprising a single polypeptide chain comprising at least two polypeptide domains connected by a polypeptide linker, the amino acid sequence of each of said polypeptide domains comprising a set of CDRs interposed between a set of FRs, each of which are respectively homologous with at least a portion of CDRs and FRs from an immunoglobulin molecule,

said binding protein being capable of binding to a preselected antigenic determinant, determined by the collective tertiary structure of said sets of CDRs held in proper information by said sets of FRs when disposed in aqueous solution, with a binding affinity at least 10^6 M^{-1}.

45. The binding protein of claim 43 or 44 having a binding affinity at least about 10^8 M^{-1}.

46. The binding protein of claim 43 or 44 having a binding affinity no less than two orders of magnitude less than the binding affinity of said immunoglobulin molecule comprising said homologous CDRs.

47. The binding protein of claim 43 or 44 wherein at least one of said polypeptide domains is peptide bonded to a biologically active amino acid sequence.

48. The binding protein of claim 43 or 44 further comprising a radioactive atom bound to said polypeptide chain.

1/31

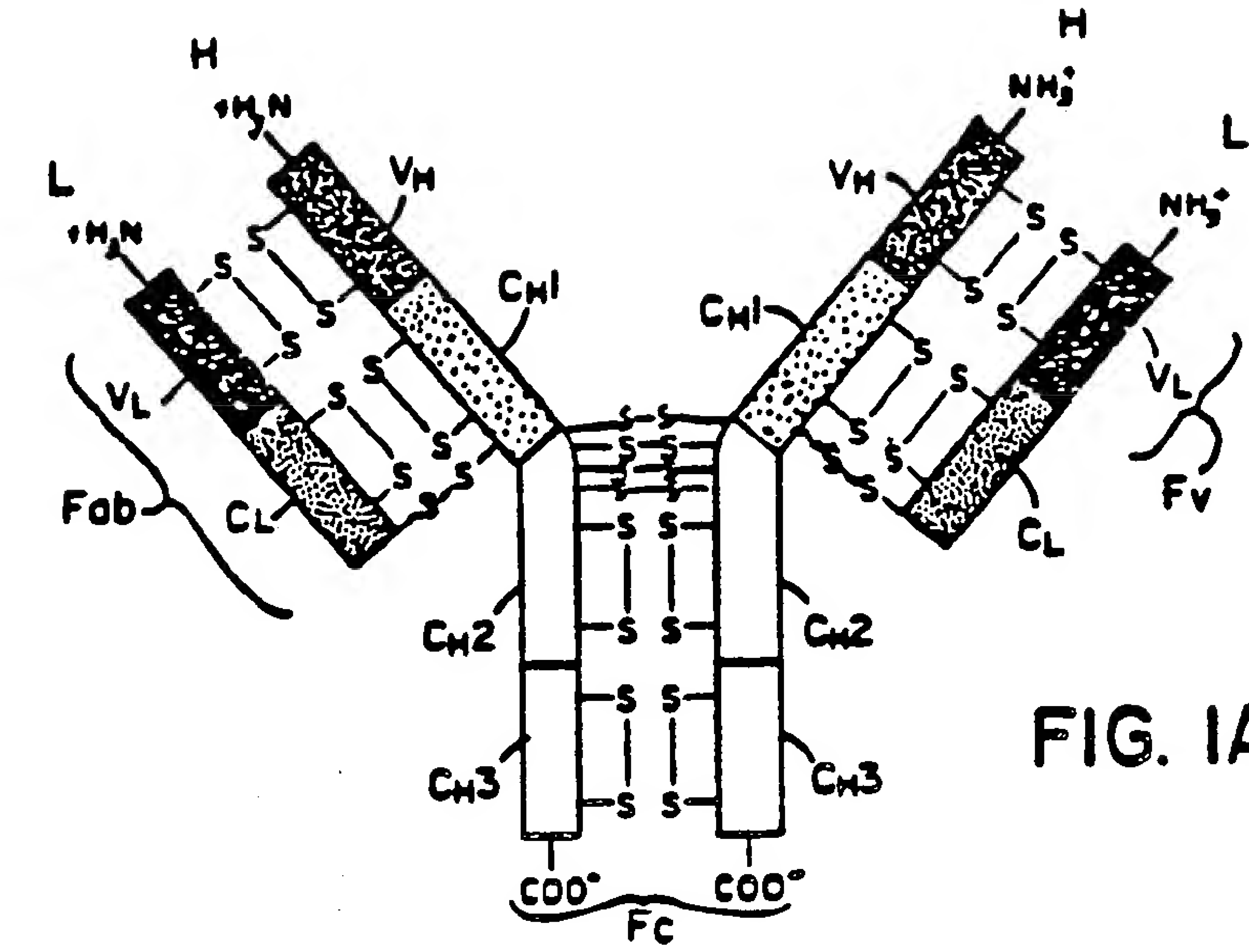


FIG. 1A

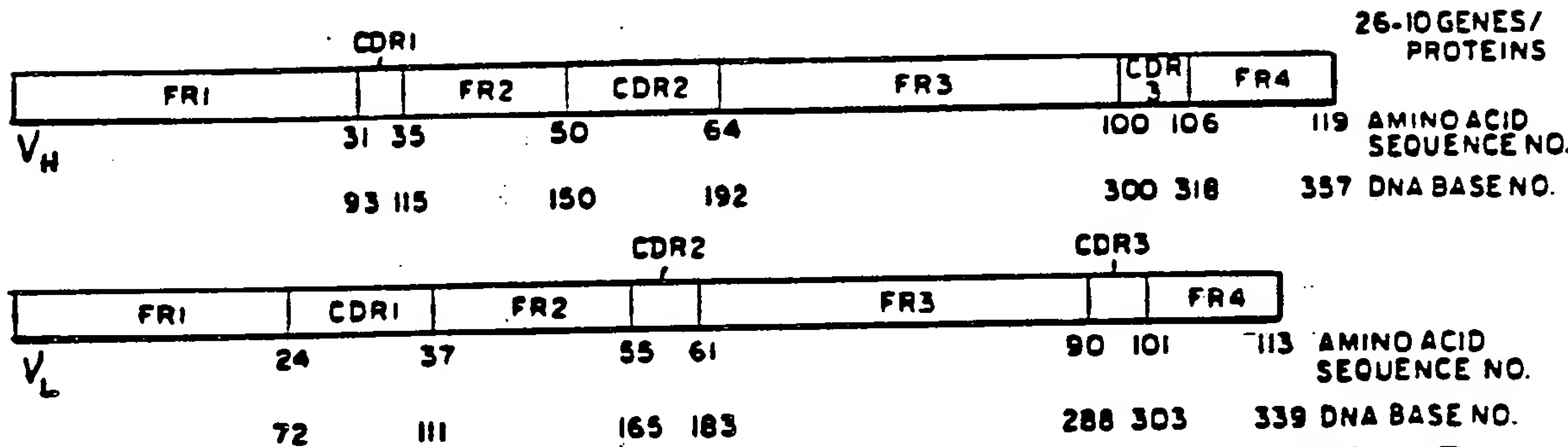


FIG. 1B

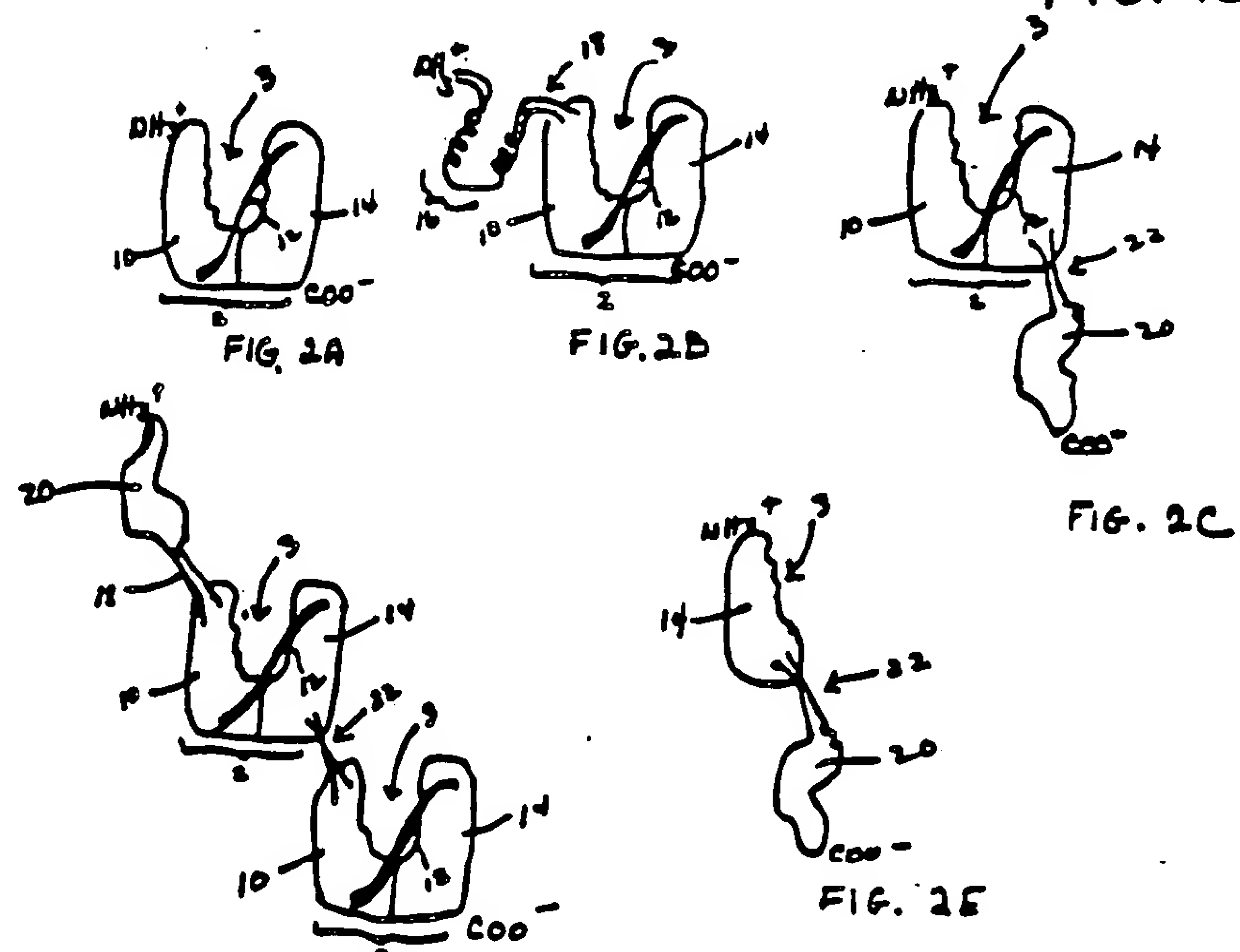


FIG. 2A

FIG. 2B

FIG. 2C

FIG. 2D

FIG. 2E

```
g-loop:
QVQLQQSGPELVEPGASVRISCTASGYTFTNYYIHWLKQRPGQGLEWIGWIYPGNGNTKYNENFKGKATLTADKSSSTAFNQISSLTSEDSAVYFCARYTHYYF   DYWGQGTTLTVSSK*

26-10:
EVQLQQSGPELVKPGASVRMSCKSSGYIFTDFYMNWVRQSHGKSLDYIGYISPYSGVTGYNQKFKGKATLTVDKSSSTAYMELRSLTSEDSAVYYCAGSSGNKWAMDYWGHGASVTVSS*

26-10/g-loop hybrid:
EVQLQQSGPELVKPGASVRMSCKSSGYtftnyyihwlkQSHGKSLewigwiypgngntkynenfkgKATLTaDKSSSTAYMELRSLTSECSAVYYCArythyyf   DYWGHGASVTVSS*
                               (cdr1-------)     (cdr2------------------) - - )                    (cdr3-----)
                              hphI        bstXI  xbaI                drаI     hincII              sacII                  nheI
newm/g-loop hybrid:                                                                                                      narI
EVQLQQSGPGLVRPSQTLSLTCTVSGStftnyyihwlkQPPGRGLewigwiypgngntkynenfkgRVTMLVDTSKNQFSLRLSSVTAADTAVYYCArythyyf   DVWGQGSLVTVSS*
                            -----------      ---------------------                                  ---------
        [newm1.............]           [newm2..]                 [newm3.....................]                  [newm4]
         avaII...........hphI          bstXI...xbaI              draI........................sacII            .............

newm:
EVQLQQSGPGLVRPSQTLSLTCTVSGSTFSNDYYTWVRQPPGRGLEWIGYVFYHGTSDDTTPLRS RVTHLVDTSKNQFSLRLSSVTAADTAVYYCARNLIAGCIDVWGQGSLVTVSS*
```

FIG. 3

3/31

```
         10        20        30        40        50        60        70
GAATTCCAAGTTCAACTGCAGCAGTCTGGTCCTGAATTGGTTAAACCTGGCGCCTCTGTGCCCATGTCCT
GluPheGluValGlnLeuGlnGlnSerGlyProGluLeuValLysProGlyAlaSerValArgMetSerC
  AsuII          BbvI     AvaII                 AhaII     HhaI
EcoRI            Fnu4HI   Sau96I                BanI      HinPI
   TaqI         PstI                          EcoRII     MstINlaIII
                                               HaeII     FspI
                                                HhaI
                                                HinPI
                                               NarI
                                               NlaIV
                                             SerFI
                                               AcyI

         80        90       100       110       120       130       140
GCAAATCCTCTGGGTACATTTTCACCGACTTCTACATGAATTGGGTTCGCCAGTCTCATGGTAAGTCTCT
ysLysSerSerGlyTyrIlePheThrAspPheTyrMetAsnTrpValArgGlnSerHisGlyLysSerLe
            RsaI    HphI         NlaIII       BstXI   NlaIII       Xba
                                                                    Ma

        150       160       170       180       190       200       210
AGACTACATCGGGTACATTTCCCCATACTCTGGGGTTACCGGCTACAACCAGAAGTTTAAAGGTAAGGCG
uAspTyrIleGlyTyrIleSerProTyrSerGlyValThrGlyTyrAsnGlnLysPheLysGlyLysAla
I            RsaI             BstEII                   DraI
eI                                 HpaII
                                 HaeIII

        220       230       240       250       260       270       280
ACCCTTACTGTCGACAAATCTTCCTCAACTGCTTACATGGAGCTCCGTTCTTTGACCTCTGAGGACTCCG
ThrLeuThrValAspLysSerSerSerThrAlaTyrMetGluLeuArgSerLeuThrSerGluAspSerA
         AccI     MboII                AluI               DdeI  HinfIFn
         HincII                    NlaIIIBbvI                        Sac
         SalI                          Fnu4HI
          TaqI

        290       300       310       320       330       340       350
CGGTATACTATTGCGCGGGCTCCTCTGGTAACAAATGGGCCATGGATTACTGGGGTCATGGCGCCTCTGT
laValTyrTyrCysAlaGlySerSerGlyAsnLysTrpAlaMetAspTyrTrpGlyHisGlyAlaSerVa
uDII          HhaIBanII      HaeIII     HaeIII                AhaII    Ma
IIAccI          FnuDII                  NcoI                  BanI
               HinPINlaIV                 NlaIII              HaeII
                                      Sau96I                   HhaI
                                        StyI                   HinPI
                                                              NarI
                                                            NlaIII
                                                              NlaIV
                                                              AcyI

        360       370
TACTGTATCCTCATAGGATCC
lThrValSerSer*amAsp
eIII           BamH1
               NlaIV
                Sau3A
               XhoII
```

FIG. 4A

4/31

```
         10        20        30        40        50        60        70
GAATTCGACGTCGTAATGACCCAGACTCCCCTGTCTCTGCCGGTTTCTCTGGGTGACCAGGCTTCTATTT
GluPheAspValValMetThrGlnThrProLeuSerLeuProValSerLeuGlyAspGlnAlaSerIleS
EcoRI AatII          HinfI          HpaII          BstEII
      AhaII                                        HphI  EcoRII
    TaqI                                                 ScrFI
      AcyI                                          MaeIII
       MaeII

         80        90       100       110       120       130       140
CTTGCCGCTCTTCCCAGTCTCTCGTCCATTCTAATGGTAACACTTACCTGAACTGGTACCTGCAAAAGGC
erCysArgSerSerGlnSerLeuValHisSerAsnGlyAsnThrTyrLeuAsnTrpTyrLeuGlnLysAl
   Fnu4HI             AvaII          MaeIII   HgiEII    BanI
       MboII            BstXI                           KpnI
                      Sau96I                            NlaIV
                                                         RsaI

        150       160       170       180       190       200       210
TGGTCAGTCTCCGAAGCTTCTGATCTACAAAGTCTCTAACCGCTTCTCTGGTGTCCCGGATCGTTTCTCT
aGlyGlnSerProLysLeuLeuIleTyrLysValSerAsnArgPheSerGlyValProAspArgPheSer
               AluI   Sau3A                                HpaII
              HindIII                                      NciISau3A
                                                           ScrFI

        220       230       240       250       260       270       280
GGTTCTGGTTCTGGTACTGACTTCACCCTGAAGATCTCTCGTGTCGAGGCCGAGGATCTCGGTATCTACT
GlySerGlySerGlyThrAspPheThrLeuLysIleSerArgValGluAlaGluAspLeuGlyIleTyrP
              RsaI    HphI     BglII       TaqIHaeIII Sau3A
                             MboII                  XhoII
                                Sau3A
                               XhoII

        290       300       310       320       330       340       350
TCTGCTCTCAGACTACTCATGTACCCCCGACCTTCGGCGGTGGCACCAAGCTCGAGATCAAACGTTGAGGATCC
heCysSerGlnThrThrHisValProProThrPheGlyGlyGlyThrLysLeuGluIleLysArg*op
   DdeI        NlaIII       HgiEII     BanI    AluI   Sau3A MaeII   BamHI
                  RsaI                 NlaIV    AvaI                NlaIV
                                                 TaqI                Sau3A
                                                XhoI                XhoII
```

FIG. 4B

5/31

```
      10        20        30        40        50        60        70
GAATTCGAACTTCAACTGCAGCAGTCTGGTCCTGAATTGGTTAAACCTGGCGCCTCTGTGCGCATGTCCT
GluPheGluValGlnLeuGlnGlnSerGlyProGluLeuValLysProGlyAlaSerValArgMetSerC
   AsuII          BbvI      AvaII                AhaII      HhaI
EcoRI             Fnu4HI    Sau96I               BanI       HinPI
    TaqI         PstI                          EcoRII     MstINlaIII
                                                HaeII      FspI
                                                 HhaI
                                                 HinPI
                                                NarI
                                                NlaIV
                                                AcyI

      80        90        100       110       120       130       140
GCAAATCCTCTGGGTACATTTTCACCAATTACTACATCCATTGGGTTCGCCAGTCTCATGGTAAGTCTCT
              CATGTAAAAGTGGTTAATGATGTAGGTAACCCAAGCGGTC
ysLysSerSerGlyTyrIlePheThrAsnTyrTyrIleHisTrpValArgGlnSerHisGlyLysSerLe
              RsaI    HphI          FokI          BstXI  NlaIII     Xba
                                                                     Ha

      150       160       170       180       190       200       210
AGACTACATCGGCTGGATCTACCCGGGTAATGGTAACACTAAGTACTACAATGAGAACTTTAAAGGTAAG
 TGATGTCTCCCACCTAGATGGGCCCATTACCATTGTGATTCATGATGTTACTCTTGAAA
uAspTyrIleGlyTrpIleTyrProGlyAsnGlyAsnThrLysTyrTyrAsnGluAsnPheLysGlyLys
I             Sau3A  AvaI        MaeIIIDdeIRsaI             DraI
eI            XhoII   HpaII                 ScaI
                     NciI
                      NciI
                     SmaI
                     XmaI

      220       230       240       250       260       270       280
GCGACCCTTACTGTCGACAAATCTTCCTCAACTGCTTACATGGAGCTGCGTTCTTTGACCTCTGAGGACT
AlaThrLeuThrValAspLysSerSerSerThrAlaTyrMetGluLeuArgSerLeuThrSerGluAspS
             AccI    MboII              AluI                DdeI  Hinf
             HincII                  NlaIIIBbvI
             SalI                       Fnu4HI
              TaqI

      290       300       310       320       330       340       350
CCGCGGTATACTATTGCGCGGGCTCCTCTGGTAACAAATGGGCCTTCGATTACTGGGGTCATGGCGCCTC
                                         GGAAGCTAATGACCCCAGTACCCC
erAlaValTyrTyrCysAlaGlySerSerGlyAsnLysTrpAlaPheAspTyrTrpGlyHisGlyAlaSe
I   AccI        HhaIBanII     MaeIII   HaeIII                 AhaII
 FnuDII          FnuDII                Sau96ITaqI             BanI
SacII           HinPINlaIV                                    HaeII
                                                               HhaI
                                                               HinPI
                                                              NarI
                                                            NlaIII
                                                              NlaIV
                                                              AcyI

      360       370
TGTTACTGTATCCTCATAGGATCC
rValThrValSerSer*am
 MaeIII          BamH1
                 NlaIV
                  Sau3A
                 XhoII
```

FIG. 4C

```
        10        20        30        40        50        60        70
GAATTCGACGTCGTAATGACCCAGACTCCGCTGTCTCTGCCGGTTTCTCTGGGTGACCAGGCTTCTATTT
GluPheAspValValMetThrGlnThrProLeuSerLeuProValSerLeuGlyAspGlnAlaSerIleS
EcoRI AatII           HinfI            HpaII        BstEII
      AhaII                                         HphI EcoRII
   TaqI                                                  SerFI
      AcyI                                           MaeIII
       MaeII

        80        90       100       110       120       130       140
CTTGCCCCTCTTCCCAGTCTATTGTGCACTCTAATGGTAACACTTACCTGGATTGGTACCTGCAAAAGCC
 AACGGCCAGAAGGGTCAGATAACACGTGAGATTACCATTGTGAATGGACCTAAC
erCysArgSerSerGlnSerIleValHisSerAsnGlyAsnThrTyrLeuAspTrpTyrLeuGlnLysAl
  Fnu4HI              HgiAI          MaeIII     EcoRII  BanI
       MboII                                    SerFI   KpnI
                                               HgiEII   NlaIV
                                                         RsaI

       150       160       170       180       190       200       210
TGGTCAGTCTCCGAAGCTTCTGATCTACAAAGTCTCTAACCGCTTCTCTGGTGTCCCGCATCGTTTCTCT
aGlyGlnSerProLysLeuLeuIleTyrLysValSerAsnArgPheSerGlyValProAspArgPheSer
             AluI    Sau3A                                 HpaII
            HindIII                                        NciISau3A
                                                           SerFI

       220       230       240       250       260       270       280
GGTTCTGGTTCTGGTACTGACTTCACCCTGAAGATCTCTCGTGTCGAGGCCGAGGATCTGGGTATCTACT
                                                 GGCTCCTAGACCCATAGATGA
GlySerGlySerGlyThrAspPheThrLeuLysIleSerArgValGluAlaGluAspLeuGlyIleTyrT
            RsaI      HphI     BglII          TaqIHaeIII Sau3A
                             MboII                      XhoII
                                Sau3A
                               XhoII

       290       300       310       320       330       340       350
ACTGCTTCCAGGGGTCTCATGTACCGTGGACCTTCGGCGGTGGCACCAAGCTCGAGATCAAACGTTGAGGATCC
TGACGAAGGTCCCCAGAGTACATGGCACCTGGAAGCCGCCACCGTGGTTCGAGCT
yrCysPheGlnGlySerHisValProTrpThrPheGlyGlyGlyThrLysLeuGluIleLysArg*op
       EcoRII    NlaIII    AvaII         BanI   AluI   Sau3A MaeII  BamHI
       SerFI        RsaI   Sau96I        NlaIV    AvaI               NlaIV
                             HgiEII                TaqI                Sau3A
                                                  XhoI               XhoII
```

FIG. 4D

10 20 30 40 50 60 70
GAATTCATGGAAGTACAACTGCAACAATCTGGGCCCGGTCTGGTACGTCCGTCTCAGACTCTGTCCCTGA
GluPheMetGluValGlnLeuGlnGlnSerGlyProGlyLeuValArgProSerGlnThrLeuSerLeuT
EcoRINlaIII RsaI ApaIHpaII RsaI DdeIHinfI
BanII MaeII Tth111I
HaeIII
NciI
NlaIV
Sau96I
Sau96I
ScrFI

80 90 100 110 120 130 140
CTTGTACCGTATCCGGATCCACCTTCTCTAACTACTACATCCATTGGGTCCGTCAACCGCCGGGTCGTGG
hrCysThrValSerGlySerThrPheSerAsnTyrTyrIleHisTrpValArgGlnProProGlyArgGl
RsaI BamH1 FokI AvaIIHincII HpaII
HpaII NlaIV NciI
NlaIV Sau96I ScrFI
Sau3A
XhoII

150 160 170 180 190 200 210
TCTCGAGTGGATCGGTTGGATTTACCCCGGTAATGGTAACACTAAGTACTACAATGAGAACTTTAAAGGC
yLeuGluTrpIleGlyTrpIleTyrProGlyAsnGlyAsnThrLysTyrTyrAsnGluAsnPheLysGly
AvaI Sau3A AvaI MaeIIIDdeIRsaI DraI N
TaqI HpaII ScaI Sp
XhoI NciI
NciI
ScrFI
ScrFI
SmaI
XmaI

220 230 240 250 260 270 280
ATGCTGGTCGACACTTCTAAGAACCAATTCTCTCTGCGTCTGTCTTCTGTTACCGCGGCTGATACTGCTG
MetLeuValAspThrSerLysAsnGlnPheSerLeuArgLeuSerSerValThrAlaAlaAspThrAlaV
laIII AccI DdeIXmnI HgaI MboII MaeIIIFnu4HI
hI HincII BbvII FnuDII
SalI SacII
TaqI

290 300 310 320 330 340 350
TGTACTACTGCGCGCGTTCCTCCGGTAATAAGTGGGCATTTGATTACTGGGGCCAGGGCTCTCTGGTCAC
alTyrTyrCysAlaArgSerSerGlyAsnLysTrpAlaPheAspTyrTrpGlyGlnGlySerLeuValTh
RsaI BssHII HpaII NlaIV BanII BstEII
FnuDII EcoRII HphI
FnuDII HaeIII MaeIII
HhaI Sau96I
HhaI ScrFI
HinPI
HinPI

FIG. 4E

360 370
CGTATCCTCTTAACTGCAG
rValSerSer*ocLeuGln
PstI

```
       10        20        30        40        50        60        70
GAATTCATGGAATCTGTTCTGACTCAGCCGCCGTCTGTATCTGGTGCACCGGGTCAACGCGTAACTATCT
GluPheMetGluSerValLeuThrGlnProProSerValSerGlyAlaProGlyGlnArgValThrIleS
EcoRI   HinfI        DdeIFnu4HI               HgiAIHpaII     FnuDII
    NlaIII       HinfI                           NciIHincII  HaeIII
      XmnI                                       ScrFI    MluI

       80        90       100       110       120       130       140
CTTGCCGTTCCTCTCAGTCTATTGTCCATTCTAATGGCAACACTTATCTGGAATGGTACCAACAACTGCC
erCysArgSerSerGlnSerIleValHisSerAsnGlyAsnThrTyrLeuGluTrpTyrGlnGlnLeuPr
           DdeI         BstXI                         BanI          Hp
                                                      KpnI          Nc
                                                      NlaIV         Sc
                                                       RsaI

      150       160       170       180       190       200       210
GGGCACCGCGCCGAAGCTGCTGATCTTTAAAGTATCTAATCGCTTCTCTGGCGTACCGGATCGATTCTCT
oGlyThrAlaProLysLeuLeuIlePheLysValSerAsnArgPheSerGlyValProAspArgPheSer
aII   FnuDII   AluI      DraI                       RsaI  ClaI
iI    HhaI     BbvI  Sau3A                            HpaII  HinfI
rFI   HinPI    Fnu4HI                                   Sau3A
 BanI                                                    TaqI
 NlaIV

      220       230       240       250       260       270       280
GTATCTAAGTCTGGCTCCTCTGCCACTCTGGCGATCACTGGTCTGCAAGCAGAAGATGAGGCCGATTACT
ValSerLysSerGlySerSerAlaThrLeuAlaIleThrGlyLeuGlnAlaGluAspGluAlaAspTyrT
   DdeI     NlaIV    BglI        Sau3A               MboII   HaeIII

      290       300       310       320       330       340       350
ACTGTTTTCAAGGCTCTCATGTACCGTGGACCTTCGGTGGTGGCACCAAGCTTACTGTACTGCGTCAGCC
yrCysPheGlnGlySerHisValProTrpThrPheGlyGlyGlyThrLysLeuThrValLeuArgGlnPr
                NlaIII  AvaII          BanI   AluI    RsaI  HgaI
                  RsaI  Sau96I         NlaIV  HindIII
                          HgiEII

      360
GTAACTGCAG
o*ocLeuGln
   PstI
HaeIII
```

FIG. 4F

```
                                                                                              FR-1
          10        20        30        40        50        60        70
GAAGTTCAACTGCAGCAGTCTGGTCCTGAATTGGTTAAACCTGGCGCCTCTGTGCGCATGTCCTGCAAATCCTCT
 E  V  Q  L  Q  Q  S  G  P  E  L  V  K  P  G  A  S  V  R  M  S  C  K  S  S
          BbvI+       AvaII                 AhaII          HhaI           MnlI+
          Fnu4HI      Sau96I                BanIMnlI+      HinPI
         PstI                             EcoRII          FspINlIIII
                                            HaeII            NspHI
                                             HhaI
                                             HinPI
                                            NarI
                                            NlaIV
                                          ScrFI

        X1          FR-2            X2
         85        95        105       115       125       135       145
GGGTACCGCCAGTCTCATGGTAAGTCTCTAGACTTTAAAGGTAAGGCGACCCTTACTGTCGACAAATCTTCCTCA
 G  Y  R  Q  S  H  G  K  S  L  D  F  K  G  K  A  T  L  T  V  D  K  S  S  S
 BanI   BstXI   NlaIII     XbaI                             AcoI     MboII-
 KpnI                                    DraI               HincII       MnlI+
 NlaIV                                                      SalI
  RsaI                                                        TaqI

             FR-3                                               X3
       160       170       180       190       200       210       220
ACTGCTTACATGGAGCTGCGTTCTTTGACCTCTGAGGACTCCGCGGTATACTATTGCGCGCGTATCGATTATTGG
 T  A  Y  M  E  L  R  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R  I  D  Y  W
              AluI              DdeI HinfI   AccI        AccII  ClaI      Nl
        NlaIIIBbvI-          MnlI+MnlI-   AccII            AccII TaqI     S
                Fnu4HI                    NspBII         BssHII
                                          SacII          HhaI
                                                          HhaI
                                                         HinPI
                                                          HinPI

       FR-4
       235       245       255       265
GGCCATGGCGCTAGCGTTACCGTGAGCTCCTAAGGATCC
 G  H  G  A  S  V  T  V  S  S  •  G  S
aIV    HaeII                  AluI DdeIBamHI
au96I   HhaI                 BanIIMstIINlaIV
HaeIII HinPI                 Bsp1286     Sau3A
  NcoI     NheI              HgiAI      XhoII
   NlaIII                    SacI
  StyI
```

FIG. 5

```
          10        20        30        40        50        60        70
GAATTCATGGCTGACAACAAATTCAACAAGGAACAGCAGAACGCGTTCTACGAGATCTTGCACCTGCCGAACCTG
 E  F  M  A  D  N  K  F  N  K  E  Q  Q  N  A  F  Y  E  I  L  H  L  P  N  L
EcoRI                                   MluI    BglII   BspMI+
                                       XmnI

         85        95       105       115       125       135       145
AACGAAGAGCAGCGTAACGGCTTCATCCAAAGCTTGAAAGACGACCCGTCTCAGAGCGCTAACCTGCTGGCAGAG
 N  E  E  Q  R  N  G  F  I  Q  S  L  K  D  D  P  S  Q  S  A  N  L  L  A  E
                               HindIII                          BspMI+
                                                        Eco47III

        160       170       180       190       200       210       220
GCCAAGAAACTGAACGACGCTCAGGCGCCGAAGAGTGATCCCGAAGTTCAACTGCAGCAGTCTGGTCCTGAATTG
 A  K  K  L  N  D  A  Q  A  P  K  S  D  P  E  V  Q  L  Q  Q  S  G  P  E  L
                         NarI                         PstI

        235       245       255       265       275       285       295
GTTAAACCTGGCGCCTCTGTGCGCATGTCCTGCAAATCCTCTGGGTACATTTTCACCGACTTCTACATGAATTGG
 V  K  P  G  A  S  V  R  M  S  C  K  S  S  G  Y  I  F  T  D  F  Y  M  N  W
         NarI      FspI

        310       320       330       340       350       360       370
GTTCGCCAGTCTCATGGTAAGTCTCTAGACTACATCGGGTACATTTCCCCATACTCTGGGGTTACCGGCTACAAC
 V  R  Q  S  H  G  K  S  L  D  Y  I  G  Y  I  S  P  Y  S  G  V  T  G  Y  N
    BstXI                XbaI                     PflMI     BstEII

        385       395       405       415       425       435       445
CAGAAGTTTAAAGGTAAGGCGACCCTTACTGTCGACAAATCTTCCTCAACTGCTTACATGGAGCTGCGTTCTTTG
 Q  K  F  K  G  K  A  T  L  T  V  D  K  S  S  S  T  A  Y  M  E  L  R  S  L
     DraI                         SalI

        460       470       480       490       500       510       520
ACCTCTGAGGACTCCGCGGTATACTATTGCGCGGGCTCCTCTGGTAACAAATGGGCCATGGATTATTGGGGTCAT
 T  S  E  D  S  A  V  Y  Y  C  A  G  S  S  G  N  K  W  A  M  D  Y  W  G  H
            SacII                                       NcoI

        535       545       555       565       575       585       595
GGTGCTAGCGTTACTGTGAGCTCTGGTGGCGGTGGGTCGGGCGGTGGTGGCTCGGGTGGCGGCGGATCCGACGTC
 G  A  S  V  T  V  S  S  G  G  G  G  S  G  G  G  G  S  G  G  G  G  S  D  V
   NheI           SacI                                          BamHI  AatII

        610       620       630       640       650       660       670
GTTGTTACCCAGACTCCGCTGTCTCTGCCGGTTTCTCTGGGTGACCAGGCTTCTATTTCTTGCCGCTCTTCCCAG
 V  V  T  Q  T  P  L  S  L  P  V  S  L  G  D  Q  A  S  I  S  C  R  S  S  Q
                                       BstEII                            PflM

        685       695       705       715       725       735       745
TCTCTGGTCCATTCTAATGGTAACACTTACCTGAACTGGTACCTGCAAAAGGCTGGTCAGTCTCCGAAGCTTCTG
 S  L  V  H  S  N  G  N  T  Y  L  N  W  Y  L  Q  K  A  G  Q  S  P  K  L  L
I    BstXI                             BspMI+                     HindIII
                                     KpnI
```

FIG. 6A-1

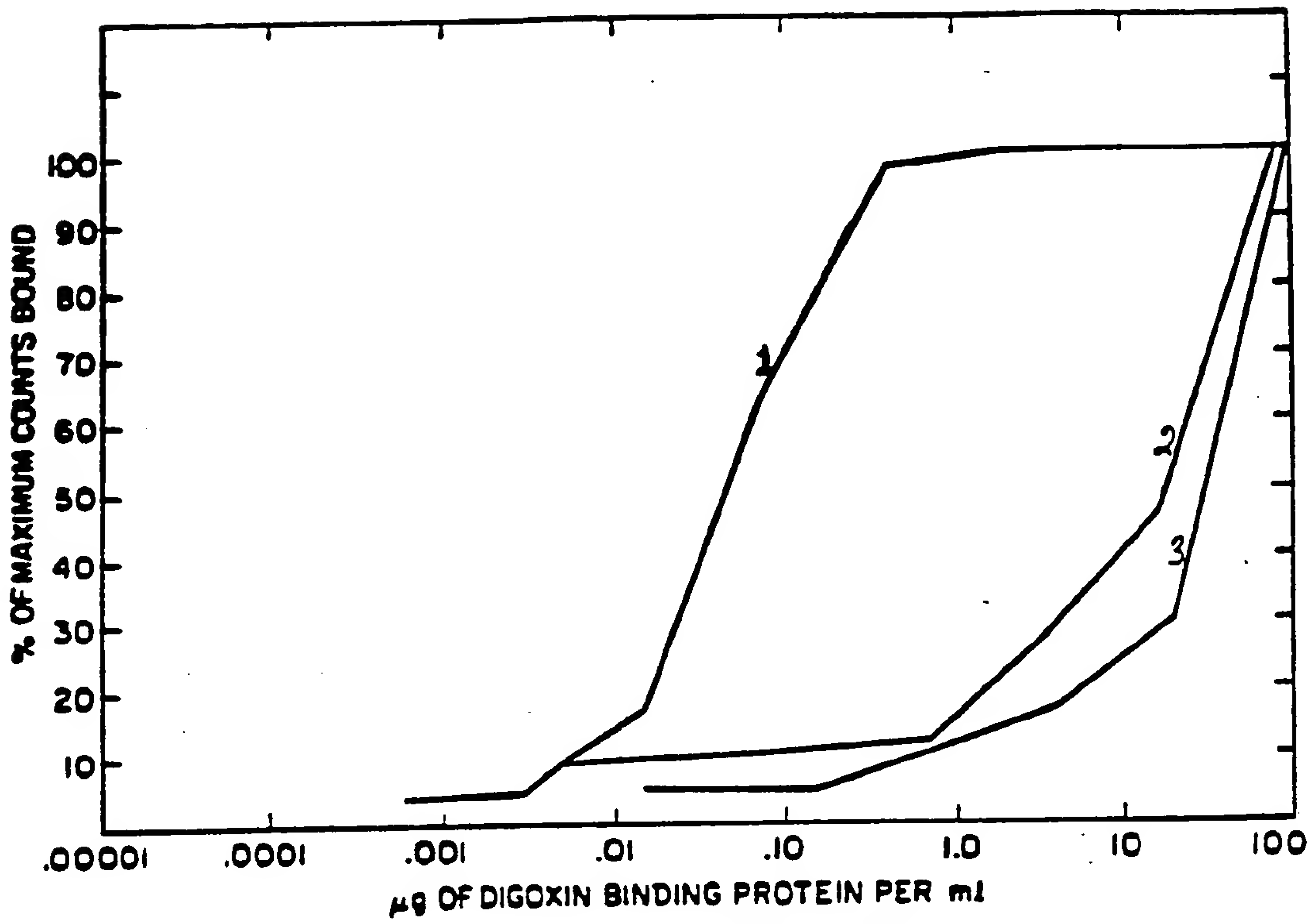


FIG. 7A

```
        760       770       780       790       800       810       820
ATCTACAAAGTCTCTAACCGCTTCTCTGGTGTCCCGGATCGTTTCTCTGGTTCTGGTTCTGGTACTGACTTCACC
 I  Y  K  V  S  N  R  F  S  G  V  P  D  R  F  S  G  S  G  S  G  T  D  F  T

        835       845       855       865       875       885       895
CTGAAGATCTCTCGTGTCGAGGCCGAAGACCTGGGTATCTACTTCTGCTCTCAGACTACTCATGTACCGCCGACT
 L  K  I  S  R  V  E  A  E  D  L  G  I  Y  F  C  S  Q  T  T  H  V  P  P  T
    BglII

        910       920       930       940
TTTGGTGGTGGCACCAAGCTCGAGATTAAACGTTAACTGCAG
 F  G  G  G  T  K  L  E  I  K  R  *
                XhoI       HpaI PstI
```

FIG. 6A-2

```
10        20        30        40        50        60
GATCCTGACGTCGTAATGACCCAGACTCCGCTGTCTCTGCCGGTTTCTCTGGGTGACCAG
 D  P  D  V  V  M  T  Q  T  P  L  S  L  P  V  S  L  G  D  Q
   AatII                                          BstEII

70        80        90        100       110       120
GCTTCTATTTCTTGCCGCTCTTCCCAGTCTCTGGTCCATTCTAATGGTAACACTTACCTG
 A  S  I  S  C  R  S  S  Q  S  L  V  H  S  N  G  N  T  Y  L
                      PflMI       BstXI

130       140       150       160       170       180
AACTGGTACCTGCAAAAGGCTGGTCAGTCTCCGAAGCTTCTGATCTACAAAGTCTCTAAC
 N  W  Y  L  Q  K  A  G  Q  S  P  K  L  L  I  Y  K  V  S  N
       BspMI+                       HindIII
   KpnI

190       200       210       220       230       240
CGCTTCTCTGGTGTCCCGGATCGTTTCTCTGGTTCTGGTTCTGGTACTGACTTCACCCTG
 R  F  S  G  V  P  D  R  F  S  G  S  G  S  G  T  D  F  T  L

250       260       270       280       290       300
AAGATCTCTCGTGTCGAGGCCGAAGACCTGGGTATCTACTTCTGCTCTCAGACTACTCAT
 K  I  S  R  V  E  A  E  D  L  G  I  Y  F  C  S  Q  T  T  H
 BglII

310       320       330       340       350       360
GTACCGCCGACTTTTGGTGGTGGCACCAAGCTCGAGATTAAACGTGGATCTGGAGGTGGC
 V  P  P  T  F  G  G  G  T  K  L  E  I  K  R  G  S  G  G  G
                              XhoI

370       380       390       400       410       420
GGATCTGGTGGAGGTGGCTCTGGTGGCGGTGGATCCGAAGTTCAATTGCAGCAGTCTGGT
 G  S  G  G  G  G  S  G  G  G  G  S  E  V  Q  L  Q  Q  S  G
                              BamHI

430       440       450       460       470       480
CCTGAATTGGTTAAACCTGGCGCCTCTGTGCGCATGTCCTGCAAATCCTCTGGGTACATT
 P  E  L  V  K  P  G  A  S  V  R  M  S  C  K  S  S  G  Y  I
                   NarI     FspI

490       500       510       520       530       540
TTCACCGACTTCTACATGAATTGGGTTCGCCAGTCTCATGGTAAGTCTCTAGACTACATC
 F  T  D  F  Y  M  N  W  V  R  Q  S  H  G  K  S  L  D  Y  I
                              BstXI             XbaI

550       560       570       580       590       600
GGGTACATTTCCCCATACTCTGGGGTTACCGGCTACAACCAGAAGTTTAAAGGTAAGGCG
 G  Y  I  S  P  Y  S  G  V  T  G  Y  N  Q  K  F  K  G  K  A
          PflMI       BstEII                 DraI

610       620       630       640       650       660
ACCCTTACTGTCGACAAATCTTCCTCAACTGCTTACATGGAGCTGCGTTCTTTGACCTCT
 T  L  T  V  D  K  S  S  S  T  A  Y  M  E  L  R  S  L  T  S
      SalI

670       680       690       700       710       720
GAGGACTCCGCGGTATACTATTGCGCGGGCTCCTCTGGTAACAAATGGGCCATGGATTAT
 E  D  S  A  V  Y  Y  C  A  G  S  S  G  N  K  W  A  M  D  Y
    SacII                                          NcoI

730       740       750       760
TGGGGTCATGGTGCTAGCGTTACTGTGAGCTCTTAACTGCAG
 W  G  H  G  A  S  V  T  V  S  S  *
```

FIG. 6B

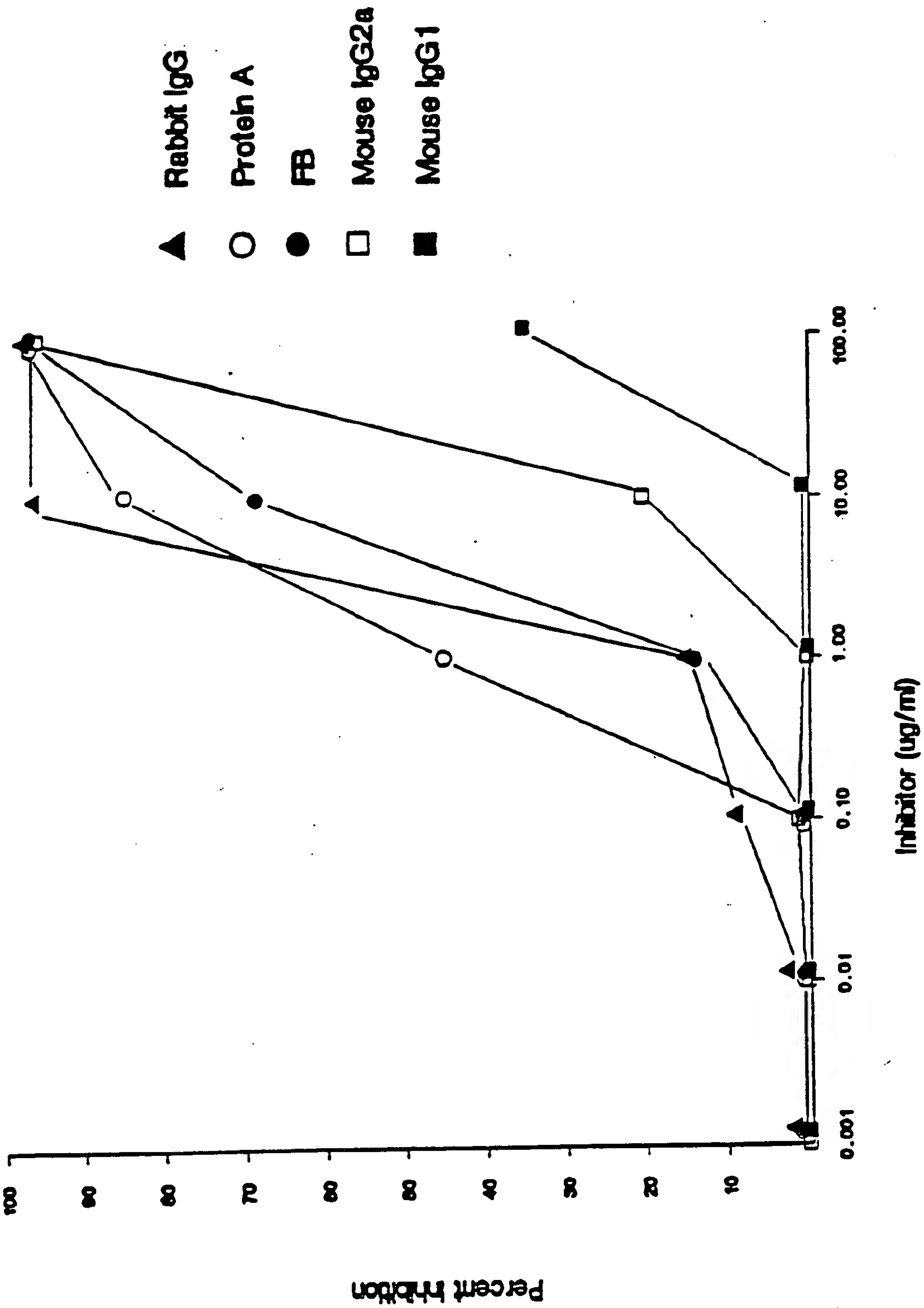
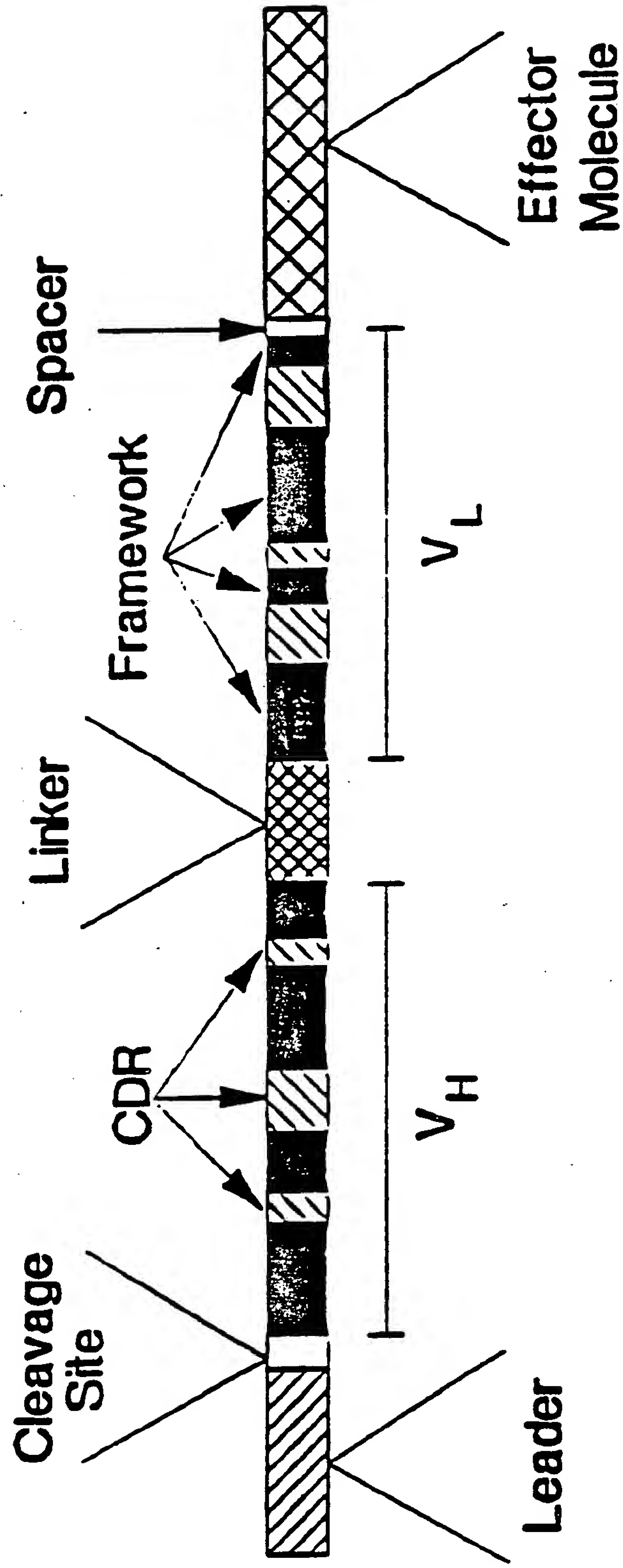

Rabbit IgG
Protein A
FB
Mouse IgG2a
Mouse IgG1
Percent Inhibition
100
90
80
70
60
50
40
30
20
10
0.001
0.01
0.10
1.00
10.00
100.00
Inhibitor (ug/ml)

FIG. 7B



Effector Molecule
Spacer
Framework
V L
Linker
CDR
V H
Cleavage Site
Leader

FIG. 8

```
        10         20         30         40         50         60
GAAGTTCAACTGGAGCAGTCTGGTCCTGGATTGGTTCGACCTTCCCAGACTCTGTCCCTG
 E  V  Q  L  E  Q  S  G  P  G  L  V  R  P  S  Q  T  L  S  L

        70         80         90        100        110        120
ACCTGCACATCCTCTGGGTACATTTTCACCGACTTCTACATGAATTGGGTTCGCCAGCCT
 T  C  T  S  S  G  Y  I  F  T  D  F  Y  M  N  W  V  R  Q  P
BspMI+                                                BstXI

       130        140        150        160        170        180
CCTGGTCGGGGTCTAGACTACATCGGGTACATTTCCCCATACTCTGGGGTTACCGGCTAC
 P  G  R  G  L  D  Y  I  G  Y  I  S  P  Y  S  G  V  T  G  Y
          XbaI                          PflMI       BstEII

       190        200        210        220        230        240
AACCAGAAGTTTAAAGGTAAGGCGACCCTTCTGGTCAACAAATCTAAGAACCAGGCTTCC
 N  Q  K  F  K  G  K  A  T  L  L  V  N  K  S  K  N  Q  A  S
       DraI

       250        260        270        280        290        300
CTGCGGCTGTCTTCTGTGACCGCTGCGGACACCGCGGTATACTATTGCGCGGGCTCCTCT
 L  R  L  S  S  V  T  A  A  D  T  A  V  Y  Y  C  A  G  S  S
                                SacII

       310        320        330        340        350        360
GGTAACAAATGGGCCATGGATTATTGGGGTCAGGGTTCTCTGGTTACTGTGAGCTCTGGT
 G  N  K  W  A  M  D  Y  W  G  Q  G  S  L  V  T  V  S  S  G
             NcoI                                     SacI

       370        380        390        400        410        420
GGCGGTGGGTCGGGCGGTGGTGGCTCGGGTGGCGGCGGATCCGACGTCGTTATGACCCAG
 G  G  G  S  G  G  G  G  S  G  G  G  G  S  D  V  V  M  T  Q
                                     BamHI AatII

       430        440        450        460        470        480
CCTCCGTCGGTTTCGGGGGCTCCTGGTCAGCGGGTTACTATTTCTTGCCGCTCTTCCCAG
 P  P  S  V  S  G  A  P  G  Q  R  V  T  I  S  C  R  S  S  Q
                                                          PflM

       490        500        510        520        530        540
TCTCTGGTCCATTCTAATGGTAACACTTACCTGAACTGGTACCAGCAACTGCCTGGTACG
 S  L  V  H  S  N  G  N  T  Y  L  N  W  Y  Q  Q  L  P  G  T
I      BstXI                             KpnI

       550        560        570        580        590        600
GCTCCGAAGCTTCTGATCTACAAAGTCTCTAACCGCTTCTCTGGTGTCCCGGATCGTTTC
 A  P  K  L  L  I  Y  K  V  S  N  R  F  S  G  V  P  D  R  F
    HindIII

       610        620        630        640        650        660
TCTGGTTCTGGTTCTGGTACTGACTTCACCCTGGCGATCACTGGTCTCCAGGCCGAAGAC
 S  G  S  G  S  G  T  D  F  T  L  A  I  T  G  L  Q  A  E  D

       670        680        690        700        710        720
GAGGCTGACTACTTCTGCTCTCAGACTACTCATGTACCGCCGACTTTTGGTGGTGGCACC
 E  A  D  Y  F  C  S  Q  T  T  H  V  P  P  T  F  G  G  G  T

       730        740        750
AAGCTCACGGTTCTGCGTTAACTGCAG
 K  L  T  V  L  R  *  L  Q
              HpaI PstI
```

FIG. 9A

```
          10        20        30        40        50        60
GAATTCGAAGTTCAACTGCAGCAGTCTGGTCCTGAATTGGTTAAACCTGGCGCCTCTGTG
 E  F  E  V  Q  L  Q  Q  S  G  P  E  L  V  K  P  G  A  S  V
  AsuII     PstI                                NarI      Fs
EcoRI

          70        80        90       100       110       120
CGCATGTCCTGCAAATCCTCTGGGTACACCTTCACCAACTATTACATCCACTGGCTTAAG
 R  M  S  C  K  S  S  G  Y  T  F  T  N  Y  Y  I  H  W  L  K
pI                                                      AflII

         130       140       150       160       170       180
CAGTCTCATGGTAAGTCTCTAGAGTGGATCGGTTGGATTTACCCGGGTAATGGTAACACT
 Q  S  H  G  K  S  L  E  W  I  G  W  I  Y  P  G  N  G  N  T
                XbaI                        SmaI

         190       200       210       220       230       240
AAGTACAATGAGAACTTTAAAGGTAAGGCGACCCTTACTGTCGACAAATCTTCCTCAACT
 K  Y  N  E  N  F  K  G  K  A  T  L  T  V  D  K  S  S  S  T
               DraI                      SalI

         250       260       270       280       290       300
GCTTACATGGAGCTGCGTTCTTTGACCTCTGAGGACTCCGCGGTATACTATTGCGCGCGT
 A  Y  M  E  L  R  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R
                                       SacII          BssHII

         310       320       330       340       350       360
TACACTCATTATTACTTCGATTATTGGGGCCATGGCGCTAGCGTTACCGTGAGCTCTGGT
 Y  T  H  Y  Y  F  D  Y  W  G  H  G  A  S  V  T  V  S  S  G
                              NcoI  NheI           SacI

         370       380       390       400       410       420
GGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCCGACGTCGTTATGACCCAG
 G  G  G  S  G  G  G  G  S  G  G  G  G  S  D  V  V  M  T  Q
                                     BamHI AatII

         430       440       450       460       470       480
ACTCCGCTGTCTCTGCCGGTTTCTCTGGGTGACCAGGCTTCTATTTCTTGCCGCTCTTCC
 T  P  L  S  L  P  V  S  L  G  D  Q  A  S  I  S  C  R  S  S
                              BstEII

         490       500       510       520       530       540
CAGTCTATCGTCCATTCTAATGGTAACACTTACCTGGAGTGGTACCTGCAAAAGGCTGGT
 Q  S  I  V  H  S  N  G  N  T  Y  L  E  W  Y  L  Q  K  A  G
          BstXI                                BspMI+
                                             KpnI

         550       560       570       580       590       600
CAGTCTCCGAAGCTTCTGATCTACAAAGTCTCTAACCGCTTCTCTGGTGTCCCGGATCGT
 Q  S  P  K  L  L  I  Y  K  V  S  N  R  F  S  G  V  P  D  R
       HindIII

         610       620       630       640       650       660
TTCTCTGGTTCTGGTTCTGGTACTGACTTCACCCTGAAGATCTCTCGTGTCGAGGCCGAG
 F  S  G  S  G  S  G  T  D  F  T  L  K  I  S  R  V  E  A  E
                                     BglII

         670       680       690       700       710       720
GATCTGGGTATCTACTACTGCTTCCAAGGGTCTCATGTACCGTGGACTTTCGGCGGTGGG
 D  L  G  I  Y  Y  C  F  Q  G  S  H  V  P  W  T  F  G  G  G

         730       740       750
ACCAAGCTCGAGATTAAACGTTAACTGCAG
 T  K  L  E  I  K  R  *  L  Q
    XhoI          HpaI  PstI
```

FIG. 9B

```
        10        20        30        40        50        60
GATCCCGAGGTTATGCTGGTTGAATCTGGTGGAGTACTGATGGAACCTGGTGGGTCCCTG
 D  P  E  V  M  L  V  E  S  G  G  V  L  M  E  P  G  G  S  L
                                 ScaI                EcoO

        70        80        90       100       110       120
AAGCTGAGCTGTGCTGCTAGCGGCTTCACGTTCTCTCGTTACGCCATGTCTTGGGTCCGT
 K  L  S  C  A  A  S  G  F  T  F  S  R  Y  A  M  S  W  V  R
 EspI        NheI                              PflMI

       130       140       150       160       170       180
CAGACTCCGGAGAAGCGTCTAGAGTGGGTCGCGACGATATCTTCTGGTGGTTCTCACACG
 Q  T  P  E  K  R  L  E  W  V  A  T  I  S  S  G  G  S  H  T
    BspMII     XbaI        NruI   EcoRV

       190       200       210       220       230       240
TTCCATCCAGACAGTGTGAAGGGTCGATTCACGATCTCTCGAGACAACGCTAAGAACACG
 F  H  P  D  S  V  K  G  R  F  T  I  S  R  D  N  A  K  N  T
                                     XhoI

       250       260       270       280       290       300
TTGTACCTGCAAATGTCTTCTCTACGTAGTGAAGATACTGCTATGTACTACTGTGCACGT
 L  Y  L  Q  M  S  S  L  R  S  E  D  T  A  M  Y  Y  C  A  R
   BspMI+          SnaBI                               ApaLI

       310       320       330       340       350       360
CCTCCACTGATCTCACTAGTTGCTGATTATGCCATGGATTATTGGGGTCATGGTGCTAGC
 P  P  L  I  S  L  V  A  D  Y  A  M  D  Y  W  G  H  G  A  S
                SpeI          NcoI                     NheI

       370       380       390       400       410       420
GTTACTGTGAGCTCTGGTGGCGGTGGGTCGGGCGGTGGTGGCTCGGGTGGCGGCGGATCG
 V  T  V  S  S  G  G  G  G  S  G  G  G  G  S  G  G  G  G  S
        SacI

       430       440       450       460       470       480
GATATCGTTATGACTCAGTCTCATAAGTTCATGTCCACTTCTGTTGGTGACCGTGTTTCT
 D  I  V  M  T  Q  S  H  K  F  M  S  T  S  V  G  D  R  V  S
EcoRV                                              BstEII

       490       500       510       520       530       540
ATCACTTGTAAGGCCAGCCAGGATGTGGGTGCTGCTATCGCATGGTATCAGCAGAAGCCC
 I  T  C  K  A  S  Q  D  V  G  A  A  I  A  W  Y  Q  Q  K  P
                PflMI                                    Sma

       550       560       570       580       590       600
GGGCAGTCTCCTAAGCTGCTGATCTACTGGGCGTCGACTCGTCATACTGGTGTCCCGGAT
 G  Q  S  P  K  L  L  I  Y  W  A  S  T  R  H  T  G  V  P  D
I                                SalI

       610       620       630       640       650       660
CGTTTCACTGGGTCCGGATCAGGTACTGATTTCACTCTGACTATTTCGAACGTTCAGTCT
 R  F  T  G  S  G  S  G  T  D  F  T  L  T  I  S  N  V  Q  S
          BspMII                           AsuII

       670       680       690       700       710       720
GATGACCTGGCTGATTACTTCTGCCAGCAATATTCCGGGTACCCTCTGACTTTCGGTGCC
 D  D  L  A  D  Y  F  C  Q  Q  Y  S  G  Y  P  L  T  F  G  A
                           SspI  KpnI                    Nae

       730       740       750
GGCACTAAACTCGAGCTGAAGTAACTGCAG
 G  T  K  L  E  L  K  *
I      XhoI           PstI
```

FIG. 9D

```
         10        20        30        40        50        60
GATCCCGAGGTTATGCTGGTTGAATCTGGTGGAGTACTGATGGAACCTGGTGGGTCCCTG
 D  P  E  V  M  L  V  E  S  G  G  V  L  M  E  P  G  G  S  L
                           ScaI                  EcoO

         70        80        90        100       110       120
AAGCTGAGCTGTGCTGCTAGCGGCTTCACGTTCTCTCGTTACGCCATGTCTTGGGTCCGT
 K  L  S  C  A  A  S  G  F  T  F  S  R  Y  A  M  S  W  V  R
 EspI        NheI                            PflMI

         130       140       150       160       170       180
CAGACTCCGGAGAAGCGTCTAGAGTGGGTCGCGACGATATCTTCTGGTGGTTCGAACACT
 Q  T  P  E  K  R  L  E  W  V  A  T  I  S  S  G  G  S  N  T
    BspMII        XbaI      NruI  EcoRV               AsuII

         190       200       210       220       230       240
TACTATCCAGACAGTGTGAAGGGTCGATTCACGATCTCTCGAGACAACGCTAAGAACACG
 Y  Y  P  D  S  V  K  G  R  F  T  I  S  R  D  N  A  K  N  T
                                    XhoI

         250       260       270       280       290       300
TTGTACCTGCAAATGTCTTCTCTACGTAGTGAAGATACTGCTATGTACTACTGTGCACGT
 L  Y  L  Q  M  S  S  L  R  S  E  D  T  A  M  Y  Y  C  A  R
   BspMI+              SnaBI                            ApaLI

         310       320       330       340       350       360
CCTCCACTGATCTCACTAGTTGCTGATTATGCCATGGATTATTGGGGTCATGGTGCTAGC
 P  P  L  I  S  L  V  A  D  Y  A  M  D  Y  W  G  H  G  A  S
              SpeI              NcoI                     NheI

         370       380       390       400       410       420
GTTACTGTGAGCTCTGGTGCCGGTGGGTCGGGCGGTGGTGGCTCGGGTGGCGGCGGATCG
 V  T  V  S  S  G  G  G  G  S  G  G  G  G  S  G  G  G  G  S
       SacI

         430       440       450       460       470       480
GATATCGTTATGACTCAGTCTCATAAGTTCATGTCCACTTCTGTTGGTGACCGTGTTTCT
 D  I  V  M  T  Q  S  H  K  F  M  S  T  S  V  G  D  R  V  S
EcoRV                                             BstEII

         490       500       510       520       530       540
ATCACTTGTAAGGCCAGCCAGGATGTGGGTGCTGCTATCGCATGGTATCAGCAGAAGCCC
 I  T  C  K  A  S  Q  D  V  G  A  A  I  A  W  Y  Q  Q  K  P
             PflMI                                        Sma

         550       560       570       580       590       600
GGGCAGTCTCCTAAGCTGCTGATCTACTGGGCGTCGACTCGTCATACTGGTGTCCCGGAT
 G  Q  S  P  K  L  L  I  Y  W  A  S  T  R  H  T  G  V  P  D
I                                SalI

         610       620       630       640       650       660
CGTTTCACTGGGTCCGGATCAGGTACTGATTTCACTCTGACTATTTCGAACGTTCAGTCT
 R  F  T  G  S  G  S  G  T  D  F  T  L  T  I  S  N  V  Q  S
          BspMII                                AsuII

         670       680       690       700       710       720
GATGACCTGGCTGATTACTTCTGCCAGCAATATTCCGGGTACCCTCTGACTTTCGGTGCC
 D  D  L  A  D  Y  F  C  Q  Q  Y  S  G  Y  P  L  T  F  G  A
                            SspI     KpnI                Nae

         730       740       750
GGCACTAAACTCGAGCTGAAGTAACTGCAG
 G  T  K  L  E  L  K  *
I     XhoI          PstI
```

FIG. 9E

```
                                        10                                      20
Met Lys Ala Ile Phe Val Leu Lys Gly Ser Leu Asp Arg Asp Leu Asp Ser Arg Leu Asp
ATG AAA GCA ATT TTC GTA CTG AAA GGT TCA CTG GAC AGA GAT CTG GAC TCT CGT CTG GAT
                                                 BglII
                                        30                                      40
Leu Asp Val Arg Thr Asp His Lys Asp Leu Ser Asp His Leu Val Leu Val Asp Leu Ala
CTG GAC GTT CGT ACC GAC CAC AAA GAC CTG TCT GAT CAC CTG GTT CTG GTC GAC CTG GCT
                                           BclI              SalI
                                        50                                      60
Arg Asn Asp Leu Ala Arg Ile Val Thr Pro Gly Ser Arg Tyr Val Ala Asp Leu Glu Phe
CGT AAC GAC CTG GCT CGT ATC GTT ACT CCC GGG TCT CGT TAC GTT GCG GAT CTG GAA TTC
                                    SmaI                                 EcoRI

Asp
GAT
```

FIG. 10A

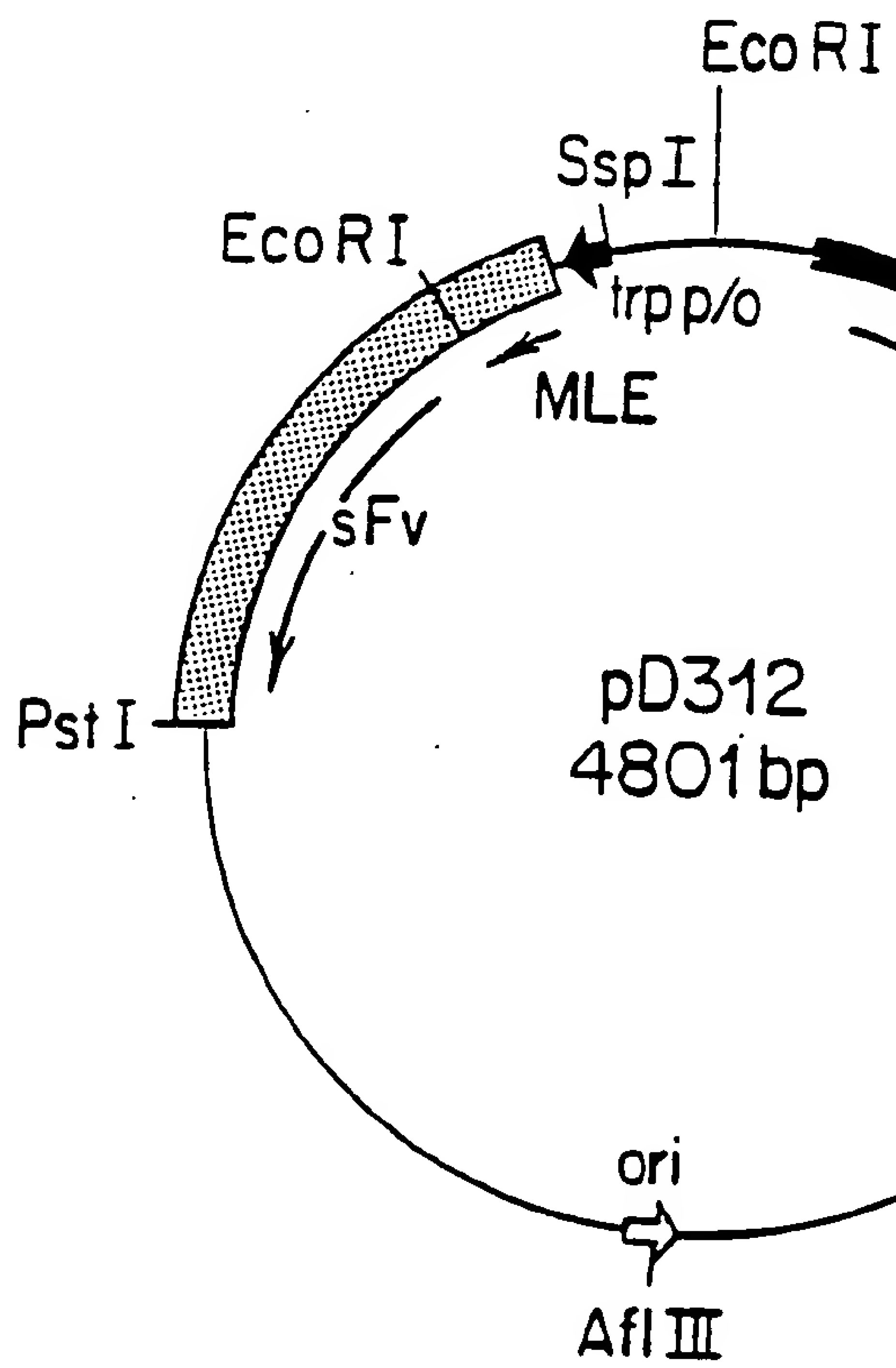


FIG. 10B

FIG. 11

D V Q L Q E S G P G L V K P S Q S L S L T C S V T G Y S I T
S G Y F W N W I R Q F P G N K L E W L G F I K Y D G S N Y G
N P S L K N R V S I T R D T S E N Q F F L K L D S V T T A T
Y Y C A G D N D H L Y F D Y W G Q G T T L T V S

G G G G S G G G G S G G G G S

Q A V V T Q E S A L T T S P G G T V I L T C R S S T G A V T
T S N Y A N W I Q E K P D H L F T G L I G G T S N R A P G V
P V R F S G S L I G D K A A L T I T G A Q T E D D A M Y F C
A L W F R N H F V F G G G T K V T V L G

FIG. 9C

21/31

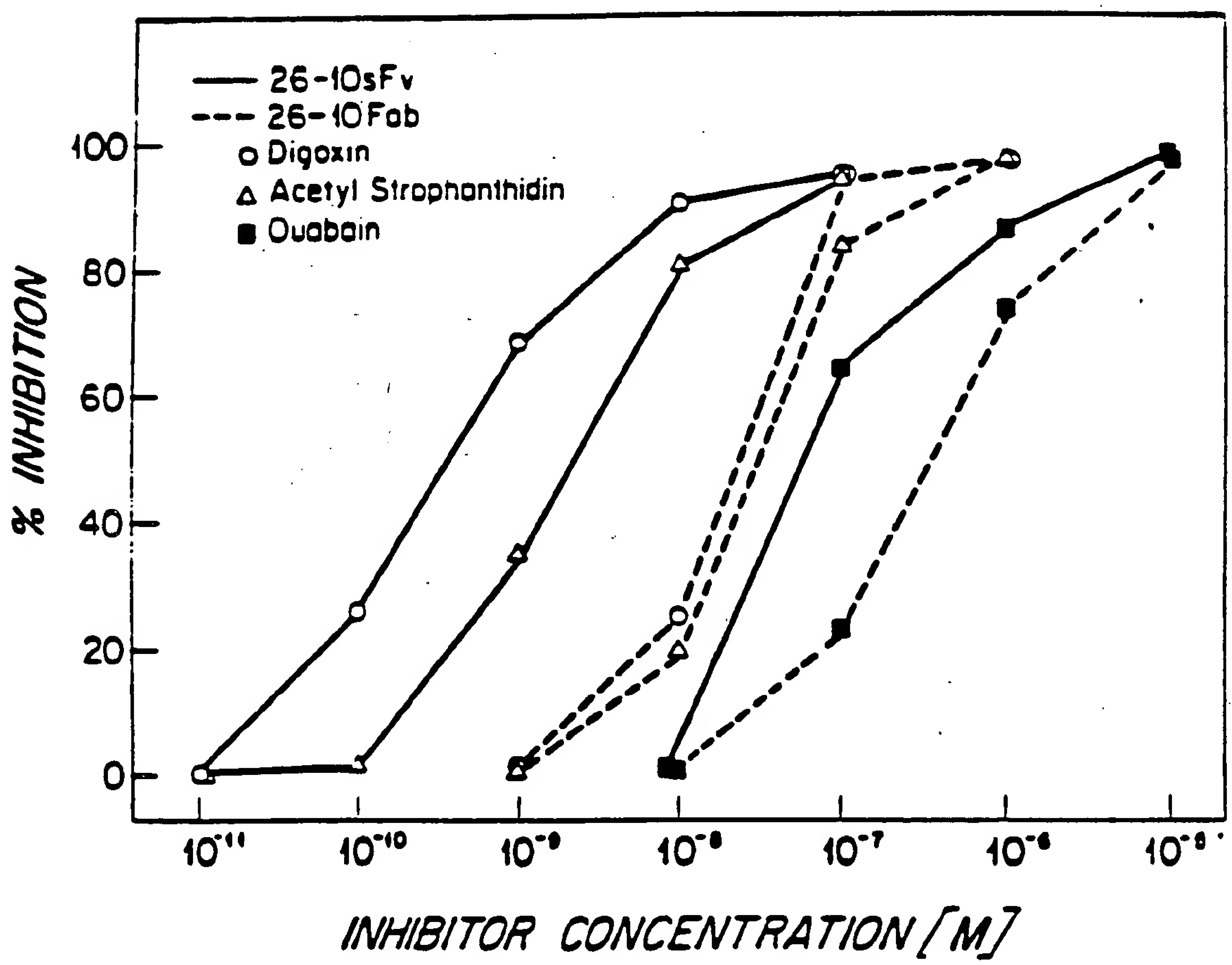

26-10sFv
26-10Fab
Digoxin
Acetyl Strophanthidin
Ouabain
100
80
60
40
20
0
% INHIBITION
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
INHIBITOR CONCENTRATION [M]

FIG. 12

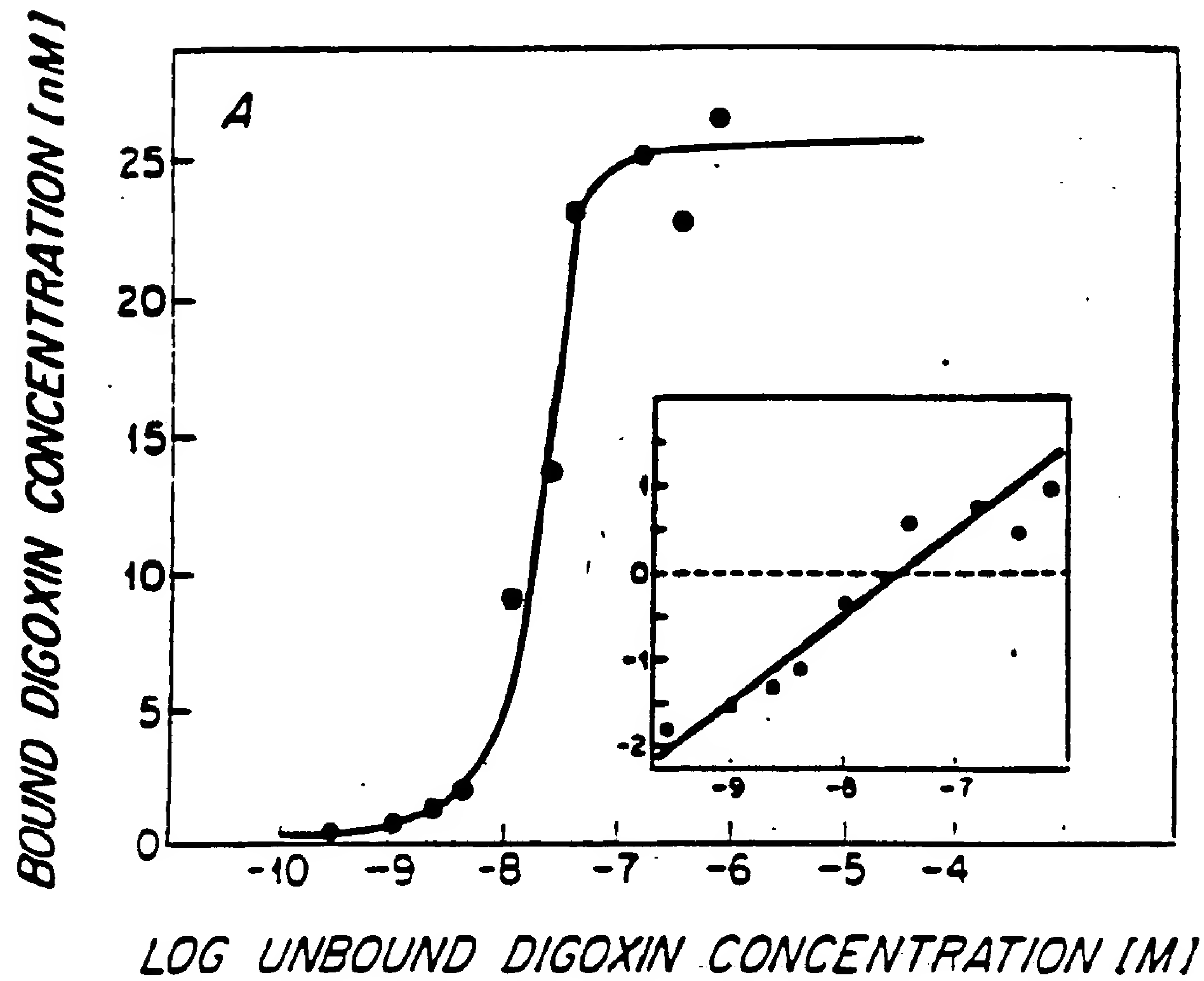


FIG. 13A

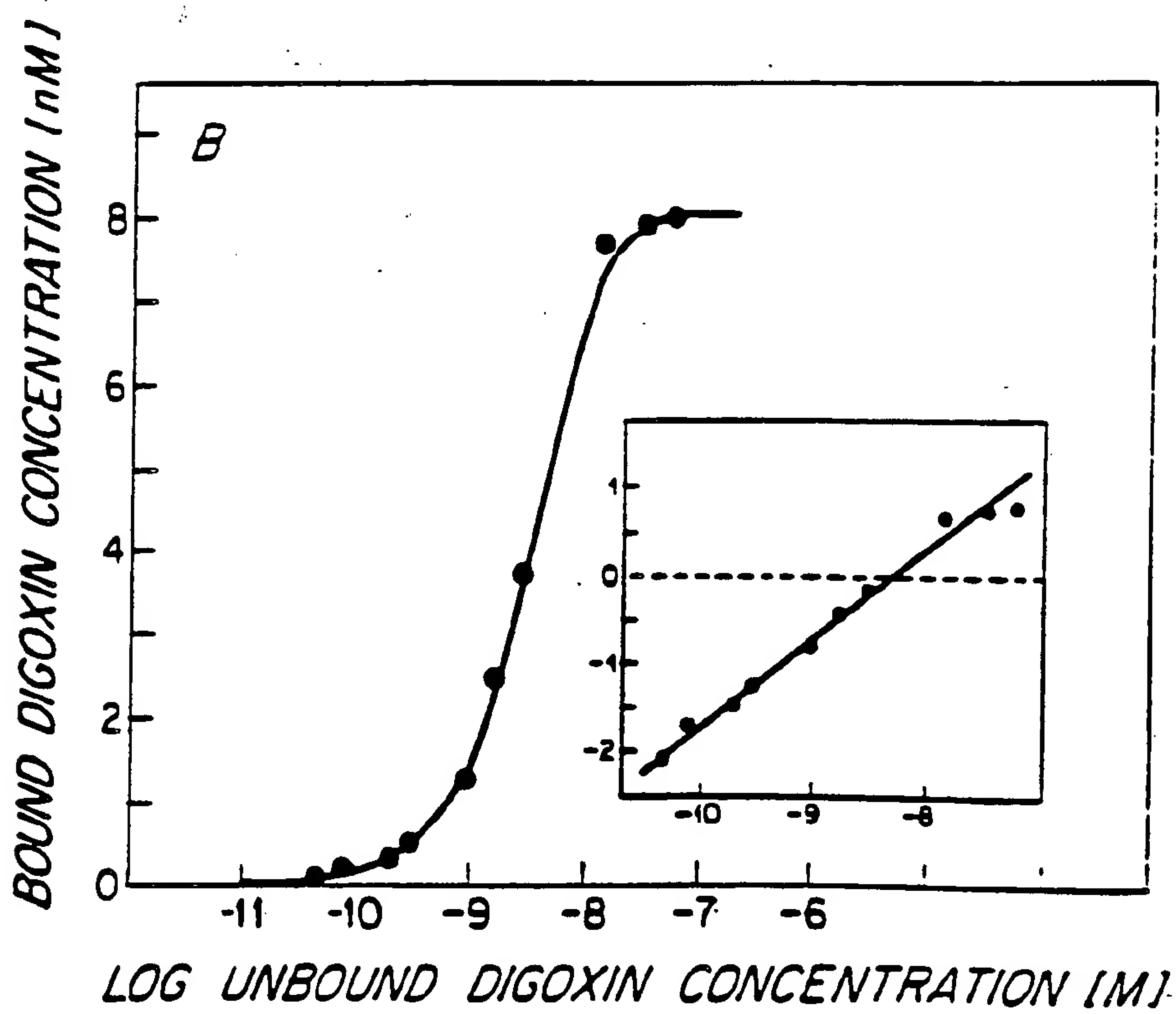


FIG. 13B

```
         10                  20                  30                  40                  50                  60
GAATTCATGGCTGACAACAAATTCAACAAGGAACAGCAGAACGCGTTCTACGAGATCTTG
 E  F  M  A  D  N  K  F  N  K  E  Q  Q  N  A  F  Y  E  I  L
EcoRI                                     MluI       BglII
                                        XmnI

         70                  80                  90                 100                 110                 120
CACCTGCCGAACCTGAACGAAGAGCAGCGTAACGGCTTCATCCAAAGCTTGAAGGATGAG
 H  L  P  N  L  N  E  E  Q  R  N  G  F  I  Q  S  L  K  D  E
BspMI+                                        HindIII

        130                 140                 150                 160                 170                 180
CCCTCTCAGTCTGCGAATCTGCTAGCGGATGCCAAGAAACTGAACGATGCGCAGGCACCG
 P  S  Q  S  A  N  L  L  A  D  A  K  K  L  N  D  A  Q  A  P
                   NheI                             FspI

        190                 200                 210                 220                 230                 240
AAATCGGATCAGGGGCAATTCATGGCTGACAACAAATTCAACAAGGAACAGCAGAACGCG
 K  S  D  Q  G  Q  F  M  A  D  N  K  F  N  K  E  Q  Q  N  A
                                                        MluI
                                                      XmnI

        250                 260                 270                 280                 290                 300
TTCTACGAGATCTTGCACCTGCCGAACCTGAACGAAGAGCAGCGTAACGGCTTCATCCAA
 F  Y  E  I  L  H  L  P  N  L  N  E  E  Q  R  N  G  F  I  Q
      BglII     BspMI+                                     H

        310                 320                 330                 340                 350                 360
AGCTTGAAGGATGAGCCCTCTCAGTCTGCGAATCTGCTAGCGGATGCCAAGAAACTGAAC
 S  L  K  D  E  P  S  Q  S  A  N  L  L  A  D  A  K  K  L  N
indIII                                NheI

        370                 380
GATGCGCAGGCACCGAAATCGGATCC
 D  A  Q  A  P  K  S  D  P
FspI                BamHI
```

FIG. 14

(BABS)-

```
              10        20        30        40        50        60        70
GGATCCGGTAACTCTGACTCTGAATGCCCGCTGAGCCACGACGGGTACTGCCTGCACGACGGTGTTTGCATGTAC
 G  S  G  N  S  D  S  E  C  P  L  S  H  D  G  Y  C  L  H  D  G  V  C  M  Y
BamHI                  BsmI+    EspI

              85        95       105       115       125       135       145
ATCGAAGCTCTGGACAAATACGCATGCAACTGCGTTGTAGGCTACATCGGTGAGCGCTGCCAGTATCGCGATCTG
 I  E  A  L  D  K  Y  A  C  N  C  V  V  G  Y  I  G  E  R  C  Q  Y  R  D  L
                      SphI                                          NruI

             160       170
AAATGGTGGGAGCTGCGTTAACTGCAG
 K  W  W  E  L  R  *
                 HpaI PstI
```

FIG. 15A

(BABS)-

```
         10        20        30        40        50        60
GGATCCGGTGGCGACCCGTCCAAGGACTCCAAAGCTCAGGTTTCTGCTGCCGAAGCTGGT
 G  S  G  G  D  P  S  K  D  S  K  A  Q  V  S  A  A  E  A  G
BamHI

         70        80        90       100       110       120
ATCACTGGCACCTGGTATAACCAACTGGGGTCGACTTTCATTGTGACCGCTGGTGCGGAC
 I  T  G  T  W  Y  N  Q  L  G  S  T  F  I  V  T  A  G  A  D
                             SalI

        130       140       150       160       170       180
GGAGCTCTGACTGGCACCTACGAATCTGCGGTTGGTAACGCAGAATCCCGCTACGTACTG
 G  A  L  T  G  T  Y  E  S  A  V  G  N  A  E  S  R  Y  V  L
 SacI                                               SnaBI

        190       200       210       220       230       240
ACTGGCCGTTATGACTCTGCACCTGCCACCGATGGCTCTGGTACCGCTCTGGGCTGGACT
 T  G  R  Y  D  S  A  P  A  T  D  G  S  G  T  A  L  G  W  T
                    BspMI+              KpnI

        250       260       270       280       290       300
GTGGCTTGGAAAAACAACTATCGTAATGCGCACAGCGCCACTACGTGGTCTGGCCAATAC
 V  A  W  K  N  N  Y  R  N  A  H  S  A  T  T  W  S  G  Q  Y
                          FspI        DraIII      BalI
                                     PflMI          BstXI

        310       320       330       340       350       360
GTTGGCGGTGCTGAGGCTCGTATCAACACTCAGTGGCTGTTAACATCCGGCACTACCGAA
 V  G  G  A  E  A  R  I  N  T  Q  W  L  L  T  S  G  T  T  E
                          DraIII      HpaI

        370       380       390       400       410       420
GCGAATGCATGGAAATCGACACTAGTAGGTCATGACACCTTTACCAAAGTTAAGCCTTCT
 A  N  A  W  K  S  T  L  V  G  H  D  T  F  T  K  V  K  P  S
 BsmI+              SpeI
  NsiI

        430       440       450       460       470       480
GCTGCTAGCATTGATGCTGCCAAGAAAGCAGGCGTAAACAACGGTAACCCTCTAGACGCT
 A  A  S  I  D  A  A  K  K  A  G  V  N  N  G  N  P  L  D  A
  NheI                                     BstEII  XbaI

        490       500
GTTCAGCAATAACTGCAG
 V  Q  Q  *
          PstI
```

FIG. 15B

(BABS)-

```
         10        20        30        40        50        60
GGATCCGGTGTACGTAGCTCCTCTCGCACTCCGTCCGATAAGCCGGTTGCTCATGTAGTT
 G  S  G  V  R  S  S  S  R  T  P  S  D  K  P  V  A  H  V  V
BamHI     SnaBI

         70        80        90       100       110       120
GCTAACCCTCAGGCAGAAGGTCAGCTTCAGTGGCTGAACCGTCGCGCTAACGCCCTGCTG
 A  N  P  Q  A  E  G  Q  L  Q  W  L  N  R  R  A  N  A  L  L
      MstII                                           BglI

        130       140       150       160       170       180
GCAAACGGCGTTGAGCTCCGTGATAACCAGCTCGTGGTACCTTCTGAAGGTCTGTACCTG
 A  N  G  V  E  L  R  D  N  Q  L  V  V  P  S  E  G  L  Y  L
            SacI          PflMI    KpnI

        190       200       210       220       230       240
ATCTATTCTCAAGTACTGTTCAAGGGTCAGGGCTGCCCGTCGACTCATGTTCTGCTGACT
 I  Y  S  Q  V  L  F  K  G  Q  G  C  P  S  T  H  V  L  L  T
           ScaI                      SalI

        250       260       270       280       290       300
CACACCATCAGCCGTATTGCTGTATCTTACCAGACCAAAGTTAACCTGCTGAGCGCTATC
 H  T  I  S  R  I  A  V  S  Y  Q  T  K  V  N  L  L  S  A  I
                                       HpaIBspMI+  Eco47III
                                                EspI

        310       320       330       340       350       360
AAGTCTCCGTGCCAGCGTGAAACTCCCGAGGGTGCAGAAGCGAAACCATGGTATGAACCG
 K  S  P  C  Q  R  E  T  P  E  G  A  E  A  K  P  W  Y  E  P
                                                NcoI

        370       380       390       400       410       420
ATCTACCTGGGTGGCGTATTTCAACTGGAGAAAGGTGACCGTCTGTCCGCAGAAATCAAC
 I  Y  L  G  G  V  F  Q  L  E  K  G  D  R  L  S  A  E  I  N
                                  BstEII

        430       440       450       460       470       480
CGTCCTGACTATCTAGATTTCGCTGAATCTGGCCAGGTGTACTTCGGTATTATCGCACTG
 R  P  D  Y  L  D  F  A  E  S  G  Q  V  Y  F  G  I  I  A  L
           XbaI            BalI

        490
TAACTGCAG
 *
   PstI
```

FIG. 15C

(BABS) -

```
         10        20        30        40        50        60
GGATCCGGTGCTGATCAGCTGACTGACGAGCAGATCGCTGAATTTAAAGAGGCTTTCTCT
 G  S  G  A  D  Q  L  T  D  E  Q  I  A  E  F  K  E  A  F  S
BamHI      BclIPvuII                       DraI

         70        80        90       100       110       120
CTGTTTGACAAAGACGGTGACGGTACCATCACTACCAAAGAGCTCGGCACCGTTATGCGC
 L  F  D  K  D  G  D  G  T  I  T  T  K  E  L  G  T  V  M  R
                    KpnI             SacI                FspI

        130       140       150       160       170       180
AGCCTTGGCCAGAACCCGACTGAAGCTGAATTGCAGGACATGATCAACGAAGTCGACGCT
 S  L  G  Q  N  P  T  E  A  E  L  Q  D  M  I  N  E  V  D  A
    BalI                                BclI          SalI

        190       200       210       220       230       240
GACGGTAACGGCACCATCGATTTTCCGGAATTTCTGAACCTGATGGCGCGCAAGATGAAA
 D  G  N  G  T  I  D  F  P  E  F  L  N  L  M  A  R  K  M  K
              ClaI     BspMII                  BssHII

        250       260       270       280       290       300
GACACTGACTCTGAAGAGGAACTGAAAGAGGCCTTCCGTGTTTTCGACAAAGACGGTAAC
 D  T  D  S  E  E  E  L  K  E  A  F  R  V  F  D  K  D  G  N
                           StuI

        310       320       330       340       350       360
GGTTTCATCTCGGCCGCTGAACTGCGTCACGTTATGACTAACCTGGGTGAAAAGCTTACT
 G  F  I  S  A  A  E  L  R  H  V  M  T  N  L  G  E  K  L  T
         EagI                                       HindIII

        370       380       390       400       410       420
GACGAAGAAGTTGACGAAATGATTCGCGAAGCTGACGTCGATGGTGACGGCCAGGTTAAC
 D  E  E  V  D  E  M  I  R  E  A  D  V  D  G  D  G  Q  V  N
              XmnI     NruI     AatII                  HpaI

        430       440       450
TACGAAGAGTTCGTTCAGGTTATGATGGCTAAGTAACTGCAG
 Y  E  E  F  V  Q  V  M  M  A  K  *
                                PstI
```

FIG. 15D

(BABS) -

```
        10        20        30        40        50        60
GGATCCGGTGGAGGCTCTCTGGGCTCTCTGACTATTGCCGAACCGGCAATGATTGCTGAA
 G  S  G  G  G  S  L  G  S  L  T  I  A  E  P  A  M  I  A  E
BamHI                                 BglI                Bsm

        70        80        90       100       110       120
TGCAAGACTCGTACCGAAGTCTTCGAGATCTCTCGTCGTCTGATCGATCGCACTAATGCC
 C  K  T  R  T  E  V  F  E  I  S  R  R  L  I  D  R  T  N  A
I+                       BglII            ClaI               Bs
                                            PvuI

       130       140       150       160       170       180
AACTTCCTGGTATGGCCGCCGTGCGTCGAGGTACAACGCTGCTCCGGGTGTTGCAACAAT
 N  F  L  V  W  P  P  C  V  E  V  Q  R  C  S  G  C  C  N  N
tXI

       190       200       210       220       230       240
CGTAACGTTCAATGTCGACCGACTCAAGTCCAGCTGCGTCCGGTCCAAGTCCGCAAAATC
 R  N  V  Q  C  R  P  T  Q  V  Q  L  R  P  V  Q  V  R  K  I
             SalI             PvuII

       250       260       270       280       290       300
GAGATTGTACGTAAGAAACCGATCTTTAAGAAGGCCACTGTTACTCTGGAAGACCATCTG
 E  I  V  R  K  K  P  I  F  K  K  A  T  V  T  L  E  D  H  L
      SnaBI

       310       320       330       340       350
GCATGCAAATGTGAGACTGTAGCGGCCGCACGTCCAGTTACTTAACTGCAG
 A  C  K  C  E  T  V  A  A  A  R  P  V  T  *
SphI                 EagI                    PstI
                    NotI
```

FIG. 15E

(BABS)-

```
        10        20        30        40        50        60
GGATCCGGTATATTCCCCAAACAATACCCAATTATAAACTTTACCACAGCGGGTGCCACT
 G  S  G  I  F  P  K  Q  Y  P  I  I  N  F  T  T  A  G  A  T
BamHI

        70        80        90       100       110       120
GTGCAAAGCTACACAAACTTTATCAGAGCTGTTCGCGGTCGTTTAACAACTGGAGCTGAT
 V  Q  S  Y  T  N  F  I  R  A  V  R  G  R  L  T  T  G  A  D

       130       140       150       160       170       180
GTGAGACATGAAATACCAGTGTTGCCAAACAGAGTTGGTTTGCCTATAAACCAACGGTTT
 V  R  H  E  I  P  V  L  P  N  R  V  G  L  P  I  N  Q  R  F

       190       200       210       220       230       240
ATTTTAGTTGAACTCTCAAATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACC
 I  L  V  E  L  S  N  H  A  E  L  S  V  T  L  A  L  D  V  T
                                                Eco47III

       250       260       270       280       290       300
AATGCATATGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACAAT
 N  A  Y  V  V  G  Y  R  A  G  N  S  A  Y  F  F  H  P  D  N
   NdeI
NsiI

       310       320       330       340       350       360
CAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAATCGATATACATTC
 Q  E  D  A  E  A  I  T  H  L  F  T  D  V  Q  N  R  Y  T  F
                                                 ClaI

       370       380       390       400       410       420
GCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGCTGGTAATCTGAGAGAAAATATC
 A  F  G  G  N  Y  D  R  L  E  Q  L  A  G  N  L  R  E  N  I

       430       440       450       460       470       480
GAGTTGGGAAATGGTCCACTAGAGGAGGCTATCTCAGCGCTTTATTATTACAGTACTGGT
 E  L  G  N  G  P  L  E  E  A  I  S  A  L  Y  Y  Y  S  T  G
                                     Eco47III       ScaI

       490       500       510       520       530       540
GGCACTCAGCTTCCAACTCTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAA
 G  T  Q  L  P  T  L  A  R  S  F  I  I  C  I  Q  M  I  S  E

       550       560       570       580       590       600
GCAGCAAGATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGAGA
 A  A  R  F  Q  Y  I  E  G  E  M  R  T  R  I  R  Y  N  R  R
                               FspI                    Bgl
```

FIG. 15F

30/31

(BABS)-

```
         10        20        30        40        50        60
GGATCCGGTGCTCCGACTTCTAGCTCTACTAAGAAAACTCAGCTTCAGCTGGAACACCTG
 G  S  G  A  P  T  S  S  S  T  K  K  T  Q  L  Q  L  E  H  L
BamHI                                          PvuII

         70        80        90       100       110       120
CTGCTGGACCTTCAGATGATCCTGAACGGTATCAACAACTACAAGAACCCGAAACTGACT
 L  L  D  L  Q  M  I  L  N  G  I  N  N  Y  K  N  P  K  L  T

        130       140       150       160       170       180
CGTATGCTGACTTTCAAATTCTACATGCCGAAGAAAGCTACCGAACTGAAACACCTTCAG
 R  M  L  T  F  K  F  Y  M  P  K  K  A  T  E  L  K  H  L  Q

        190       200       210       220       230       240
TGCCTGGAAGAAGAACTGAAGCCGCTGGAGGAAGTACTGAACCTGGCTCAGTCTAAAAAC
 C  L  E  E  E  L  K  P  L  E  E  V  L  N  L  A  Q  S  K  N
                                 ScaI

        250       260       270       280       290       300
TTCCACCTGCGTCCGCGTGACCTGATCAGCAACATCAACGTAATCGTTCTAGAACTTAAA
 F  H  L  R  P  R  D  L  I  S  N  I  N  V  I  V  L  E  L  K
                     BclI                    XbaI

        310       320       330       340       350       360
GGCTCTGAAACTACCTTCATGTGCGAATACGCTGACGAAACTGCTACCATCGTAGAATTT
 G  S  E  T  T  F  M  C  E  Y  A  D  E  T  A  T  I  V  E  F

        370       380       390       400       410       420
CTGAACCGTTGGATCACCTTCTGCCAGTCTATCATCTCTACTCTGACTTAACTGCAG
 L  N  R  W  I  T  F  C  Q  S  I  I  S  T  L  T  *
                                                    PstI
```

FIG. 15G

```
(BABS)-

          10        20        30        40        50        60
GGATCCGGTGCTGACAACAAATTCAACAAGGAACAGCAGAACGCGTTCTACGAGATCTTG
 G  S  G  A  D  N  K  F  N  K  E  Q  Q  N  A  F  Y  E  I  L
BamHI                                      MluI        BglII
                                         XmnI

          70        80        90       100       110       120
CACCTGCCGAACCTGAACGAAGAGCAGCGTAACGGCTTCATCCAAAGCTTGAAGGATGAG
 H  L  P  N  L  N  E  E  Q  R  N  G  F  I  Q  S  L  K  D  E
 BspMI+                                      HindIII

         130       140       150       160       170       180
CCCTCTCAGTCTGCGAATCTGCTAGCGGATGCCAAGAAACTGAACGATGCGCAGGCACCG
 P  S  Q  S  A  N  L  L  A  D  A  K  K  L  N  D  A  Q  A  P
                     NheI                        FspI

         190       200       210       220       230       240
AAATCGGATCAGGGGCAATTCATGGCTGACAACAAATTCAACAAGGAACAGCAGAACGCG
 K  S  D  Q  G  Q  F  M  A  D  N  K  F  N  K  E  Q  Q  N  A
                                                       MluI
                                                     XmnI

         250       260       270       280       290       300
TTCTACGAGATCTTGCACCTGCCGAACCTGAACGAAGAGCAGCGTAACGGCTTCATCCAA
 F  Y  E  I  L  H  L  P  N  L  N  E  E  Q  R  N  G  F  I  Q
      BglII    BspMI+                                      H

         310       320       330       340       350       360
AGCTTGAAGGATGAGCCCTCTCAGTCTGCGAATCTGCTAGCGGATGCCAAGAAACTGAAC
 S  L  K  D  E  P  S  Q  S  A  N  L  L  A  D  A  K  K  L  N
indIII                                 NheI

         370       380
GATGCGCAGGCACCGAAATAACTGCAG
 D  A  Q  A  P  K  *
  FspI               PstI
```

FIG. 15H

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/01737

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C07K 13/00, C12P 21/00, C12N 15/00, C07H 15/12
U.S. CL.: 530/287, 435/68, 435/172.3, 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
U.S.	530/387,388 935/6,9,10,11,15,22,23,24,59,60 435/68,170,172.3, 240.1 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

Chemical Abstract Data Base (CAS) 1967-1988; BIOSIS DATA BASE 1969-1988 Keywords: antigen, binding, site, synthetic, biosynthesis, See attachment.

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	U.S., A, 4,642,334 (MOORE ET AL) 10 February 1987. See abstract and columns 2, 3, 25 and 26.	1-48
Y	BIOCHEMISTRY, Volume 17 issued 1978, September (Washington, D.C., U.S.A.), (M.S. ROSEMBLATT ET AL.) "Isolation of an active variable-domain fragment from a homogeneous rabbit antibody heavy chain, physiochemical and immunological properties", See pages 3877-3882, See particularly page 3877.	1-48
Y	BIOCHEMISTRY, Volume 19, issued 1980, August (Washington, D.C. U.S.A.), (P.H. EHRLICH ET AL), "Isolation of an active heavy-chain variable domain from a homogeneous rabbit antibody by cathepsin B digestion of the aminoethylated heavy chain" See pages 4091-4096. See particularly page 4091.	1-48

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 August 1988	08 SEP 1988
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Richard C Peet Richard Peet

Form PCT/ISA/210 (second sheet) (Rev.11-87)

Attachment to PCT/ISA/210
II. Field Searched

Keywords Continued

immunoglobulin, specificity, variable, region, domain, chimeric, heavy, light, Fv, antibody, antibodies, cancer, tumor, treatment.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCES, U.S.A., Volume 81, issued 1984, November (Washington, D.C., U.S.A.), (MORRISON ET AL.), "Chimeric human antibody molecules: mouse antigen binding domains with human constant region domains", See pages 6851-6855. See particularly pages 6851 and 6855.	1-48
Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCES U.S.A., Volume 84, issued 1987, January (Washington, D.C., U.S.A.), (L.K. SUN ET AL "Chimeric antibody with human constant regions and mouse variable regions directed against carcinoma-associated antigen 17-A", See pages 214-218. See particularly page 214.	1-48
Y	PROCEEDINGS NATIONAL ACADEMY SCIENCES U.S.A., Volume 82, issued 1985, May (Washington, D.C., U.S.A.), (S. OHNO ET AL), "Antigen-binding specificities of antibodies are primarily determined by seven residues of V_H", See pages 2945-2949. See particularly page 2945.	1-48
Y	CHEMICAL ABSTRACTS, Volume 107, No. 5, issued 1987, August 3 (Columbus, Ohio, U.S.A.), A.P. RICHARDSON ET AL., "The radiolabeling of antibodies for use in tumor location and treatment", See page 330 column 1, the abstract no. 35697n, Med. Sci. Res. 1987, 15(7), 343-7 (Eng.)	1-48
Y	CHEMICAL ABSTRACTS, Volume 105, No. 5, issued 1986, August 4 (Columbus, Ohio, U.S.A.), R. ARNON "Immuno targeted chemotherapy in cancer research", See page 1, column 2, abstract no. 34903y. Proc. FEBS Congr. 16th 1984 (Pub. 1985). B, 565-70 (Eng.)	1-48

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	CHEMICAL ABSTRACTS, Volume 106, No. 13, issued 1987, March 30 (Columbus, Ohio U.S.A.), H.J. THIESEN ET AL "Selective Killing of human bladder cancer cells by combined treatment with A and B chain ricin antibody conjugates", See page 37, column 1, abstract no. 95759g. Cancer Res., 1987, 47(2), 419-423 (Eng.)	1-48
Y	CHEMICAL ABSTRACTS, Volume 105, No. 11, issued 1986, September 15 (Columbus, Ohio, U.S.A.), M.S. NEUBERGER "Novel antibodies by DNA transfection", see page 475, column 2, abstract no. 95609d, Adv. Immunopharmacol. 3., Proc. Int. Conf. 3rd 1985 (Pub. 1986), 317-327 (Eng.)	1-48
Y	WO, A, WO 86/0090 DAW 3 January 1986 (03.01.86) See pages 1-16, See particularly pages 2-9.	1-48
Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCES, U.S.A. Volume 81, issued 1984, June (Washington, D.C. U.S.A.), (S. CABILLY ET AL.) "Generation of antibody activity from immunoglobulin polypeptide chains produced in Escherichia coli" See pages 3273-3277. See particularly pages 3273 and 3275.	1-48
X Y	WO, A, WO 86/01533 DAW 13 March 1986 (13.03.86) See pages 1-32. See particularly pages 3-8.	11 1-10, 12-48
E	U.S. A, 4,704,692 (R.C. LADNER) 3 November 1987. See abstract and columns 2 and 14.	1-48